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THE INTERACTION BETWEEN EXOGENOUS NORADRENALINE AND TRANSMURAL NERVE STIMULATION IN THE CANINE SAPHENOUS VEIN

by

MANOHARA P.J. SENARATNE

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

IN

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EDMONTON, ALBERTA

FALL 1984

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This thesis is dedicated to my wife, Savitri, and to my parents who went through a lot of hardship in helping me achieve this goal.

ABSTRACT

The present investigation was undertaken to study the interaction between contractions produced by exogenous noradrenaline (NA) and contractions produced by transmural nerve stimulation (TNS) in isolated canine saphenous vein rings/strips. Four protocols were carried out. Protocol 1.1: Response to a single dose of exogenous NA was determined as a control. After washing, TNS was applied to produce a contraction between 10-90 per cent of the control value and the same dose of NA applied against this background and the response determined. Finally, after washing, control response with NA was repeated. The observed contraction to NA against a background of TNS was significantly less than the expected contraction (78 observations in 12 veins, $p < 0.001$). Thus the response to exogenous NA was inhibited by background TNS. Further, the inhibition appeared to be greater with increasing magnitude of background TNS. Protocol 1.2: The reciprocal of Protocol 1.1 was done here. The response to TNS was determined against a background contraction by exogenous NA. The observed contraction to TNS was significantly greater than the expected contraction (71 observations in 12 veins, $p < 0.001$). Thus, background exogenous NA was found to enhance the response to TNS. Protocol 1.3: The inhibition of the NA contraction observed in Protocol 1.1 was not observed in the presence of guanethidine or diltazem hydrochloride in a sufficient concentration to block the contractile response to TNS while maintaining the electrical current. Protocol 1.4: The response to exogenous NA, added against background contractions produced by tyramine, methooxamine, phenylephrine and histamine, was investigated here. The inhibitory phenomenon observed in Protocol 1.1 was no longer evident with any of the above agonists. Protocol 1.5: The inhibition of exogenous NA contraction was not significantly altered by propranolol (10^{-5} mol/l), indomethacin (10^{-5} mol/l), cimetidine (10^{-5} mol/l) and aminophylline (10^{-5} mol/l). Protocol Two: Protocol 1.1 and Protocol 1.2 carried out in a superfused saphenous vein strip (following labelling of NA stores with ^{3}H-noradrenaline) demonstrated inhibition of the TNS induced release of ^{3}H-noradrenaline by exogenous NA. Protocol Three: The response to TNS in a vein ring pre-contracted with prostaglandin $F_{2\alpha}$ (10^{-5} mol/l) following sympathetic (guanethidine 10^{-4} mol/l, phenoxybenzamine 2 x 10^{-5} mol/l) and muscarinic blockade (atropine 5 x 10^{-6} mol/l) was examined here. TNS applied as intermittent trains of stimuli (9V, 1.0 ms, 1-32 Hz for 30 seconds) produced a frequency dependent relaxation (maximum relaxation, mean 3.28±0.18 SEM g, n=17). This relaxation was not significantly altered by tetrodotoxin (10^{-6} mol/l), cimetidine, aminophyline, indomethacin, catalase or ascorbic acid. The relaxation was not present in rings following cold storage of the saphenous veins at 4°C for 9 days. The relaxation was also abolished by ouabain (2 x 10^{-4} mol/l) and zero-K^{+} "Krebs-bicarbonate" buffer solution. Protocol Four: Sub-threshold (for contraction) concentrations of NA was found to potentiate the response to trains of TNS (9V, 8 Hz, 0.3 ms for 5 seconds) in a concentration dependent manner (maximum potentiation; mean 246.2±33.2% SEM, of control). The present study indicates (1) Background TNS inhibits the contractions produced by exogenous NA. (2) TNS produces a non-adrenergic, non-cholinergic relaxation which could be mediated by a tetrodotoxin-resistant nerve or a direct (non-neurogenic) effect of TNS. This relaxatory phenomenon may

account for the inhibition of NA contraction mentioned above. (3) Background exogenous NA (including sub-threshold concentrations) potentiate the responses to TNS. (4) Thus, there is a significant interaction between the effects of exogenous NA and TNS in the canine saphenous vein.

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INTRODUCTION

The sympathetic nervous system exerts its effects in the body via the catecholamines noradrenaline, adrenaline and dopamine(1). Noradrenaline and adrenaline, the predominant neurotransmitters in the peripheral sympathetic nerves, are released at two major sites: the post-ganglionic sympathetic nerve endings and the adrenal glands which contain specialised post-ganglionic neuronal cell bodies that secrete these two hormones. Noradrenaline is the predominant catecholamine released at the former site while more adrenaline than noradrenaline is secreted at the latter site. In vascular smooth muscle both noradrenaline and adrenaline exert their excitatory actions through stimulation of the α-receptors located on the smooth muscle cell membrane. The action of noradrenaline on isolated smooth muscle has been investigated in the tissue bath by two methods: by the addition of noradrenaline from outside into the tissue bath (noradrenaline from outside, referred to as exogenous noradrenaline) and by transmural nerve stimulation. The technique of transmural nerve stimulation introduced by Paterson(2), leads to the electrical activation of intramural nerves present in the isolated tissue resulting in the release of the neurotransmitters from the nerve endings which act on the smooth muscle present in the tissue. In the case of sympathetic nerves in vascular smooth muscle noradrenaline is released, leading to contraction of the muscle. This noradrenaline, released by transmural nerve stimulation, is referred to as endogenous noradrenaline to differentiate it from the exogenous noradrenaline.

Until recently, both exogenous and endogenous noradrenaline were believed to exert contractile effects in vascular smooth muscle through a single population of α-receptors(3). Evidence emerged in the early 1970's that the α-receptors located at pre-synaptic sites might be pharmacologically different from those located at post-synaptic sites(4,5). Drugs such as clonidine and α-methylnoradrenaline were found to be more effective at the pre-synaptic α-receptor, in causing inhibition of release of noradrenaline from the sympathetic nerve endings than on the post-synaptic α-receptor sites on the smooth muscle membrane mediating muscle contraction. On the other hand, drugs such as methoxamine and phenylephrine were far more effective as agonists at the post-synaptic membrane, than at the pre-synaptic membrane. Alpha antagonists also showed a similar differential sensitivity at the pre- and post-synaptic receptor sites. In order to compare the potency of different drugs at the two receptor sites, the effectiveness of both α-agonists and α-antagonists at the pre- and post-synaptic α-receptors was expressed as a ratio. This pre/post ratio varied from 0.01 to 100 with different drugs indicating a wide spectrum of differential sensitivity at the two receptors(6). These findings led to the classification of post-synaptic receptors as α_1 and the pre-synaptic receptors as α_2.

However, difficulties arose with this terminology, as alpha-receptors demonstrating the pharmacological characteristics of α_2 receptors, were found outside the pre-synaptic membrane of sympathetic nerve endings. For instance, α-receptors inhibiting the release of acetylcholine, were found at parasympathetic nerve endings: the location of these receptors would be post-synaptic in relation to sympathetic nerve endings and pre-synaptic in relation to the parasympathetic nerve endings containing the receptors. α_2 receptors in the central nervous system also posed a problem in this pre-synaptic/post synaptic classification of the α-receptor sub-types. Thus it was proposed that the sub-types of the alpha receptors be classified as α_1 and α_2 on a functional basis rather than on an "anatomical" basis(6). Nevertheless, the α-receptors on the vascular smooth muscle cell membrane were considered to be of a single (α_1) type. This concept was questioned by the findings of Bentley, Drew and Whiting (7) in an in vivo study using pithed rats and anaesthetised cats which demonstrated that two types of α-receptors mediated the pressor responses of the adrenergic agonists used: one type was selectively stimulated by phenylephrine and blocked by prazosin (α_1-receptor); the other was stimulated by noradrenaline and blocked by phentolamine, but resistant to prazosin (α_2- receptor). Further in vivo studies by Drew and Whiting(8), Docherty, McDonald and McGrath(9) and Timmemans, Kwa and Van Zwieten(10,11) provided more definitive evidence for the existence of both α_1 and α_2 receptors in vascular smooth muscle. Later, De Mey and Vanhoutte demonstrated evidence for the existence of both α_1 and α_2 post-synaptic adrenoceptors in vitro in canine arteries and veins(12).

Possible locations for these two types of post-synaptic α-receptors on the smooth muscle cell were suggested by the elegant studies of Hirst and Neild in the guinea-pig intestinal sub-mucosal arterioles(13,14). The response of a 300-400 μm segment of an arteriole, to iontophoretically applied noradrenaline was investigated in this study. Two distinct types of responses were elicited by the drug at different sites of application. At some sites a localised constriction of the arteriole at the site of injection was observed. This response was not associated with a change in membrane potential, and was the more common response observed. At other sites of application, a depolarization similar to an excitatory junction potential was observed. This led to a generalised constriction of the arteriolar segment if an action potential was initiated by this depolarization. The former type of response was abolished by phentolamine but the latter type of response could be detected in the presence of phentolamine. Transmural nerve stimulation applied to this preparation produced excitatory junction potentials: increasing the stimulus strength, increased the magnitude of the junction potential leading to the triggering of an action potential and a generalised constriction. The junction potentials were not reduced by phentolamine, tolazoline or prazosin. Thus, the depolarization and generalised constriction produced at occasional sites by iontophoretically applied noradrenaline seemed to mimic the effects of transmural nerve stimulation. A mapping procedure using fluorescence microscopy to visualise the nerves was employed to find the anatomical relationship of the adrenergic nerves to the different sites producing the two types of responses to iontophoretically applied noradrenaline. This demonstrated that the sites at which depolarization was elicited by iontrphoretically applied noradrenaline were restricted to regions close to the sympathetic nerves supplying the muscle. No changes in membrane potential were detected when the point of application of the noradrenaline was greater than 10 μm from a fluorescent nerve. Although a few depolarization responses were detected at regions 5-10 μm distant from a nerve, the majority of sensitive areas were within 5 μm of a fluorescent nerve. Thus the concept of intrajunctional alpha-receptors located at the sympathetic nerve endings and extrajunctional alpha-receptors located outside the nerve endings (i.e. outside the synaptic cleft) emerged (Fig. 1). However, Hirst and Neild concluded that the intrajunctional receptor may not be an α-type receptor because of its resistance to α-antagonists.

Based on the above findings it could be postulated that endogenous noradrenaline (released at the sympathetic nerve endings into the junctional cleft) would exert a greater portion of its effects through intrajunctional α-receptors and exogenous noradrenaline would exert a greater portion of its effects through extrajunctional α-receptors. In isolated vascular smooth muscle experiments in tissue baths, endogenous and exogenous noradrenaline would be equivalent to transmural nerve stimulation (assuming a predominantly sympathetic innervation) and noradrenaline added into the bath from outside respectively. Although the individual effects of transmural nerve stimulation (TNS) and exogenous noradrenaline in isolated blood vessels have been extensively investigated, not much information is available about the interaction between the two effects. This interaction has been studied with respect to the pre-synaptic inhibition produced by exogenous noradrenaline on the release of endogenous noradrenaline via TNS(15). However, little attention has been paid to the post-synaptic aspect of this interaction(16). This is probably due to the fact that the differences in the mediation of the responses of the two modes of stimulation on the post-synaptic membrane became apparent only during the last few years with the discovery of both α_1 and α_2 receptors in smooth muscle.

The present investigation was undertaken to study the interaction between exogenous noradrenaline and transmural nerve stimulation in vascular smooth muscle; different concentrations of exogenous noradrenaline and different frequencies of TNS were utilised to span the

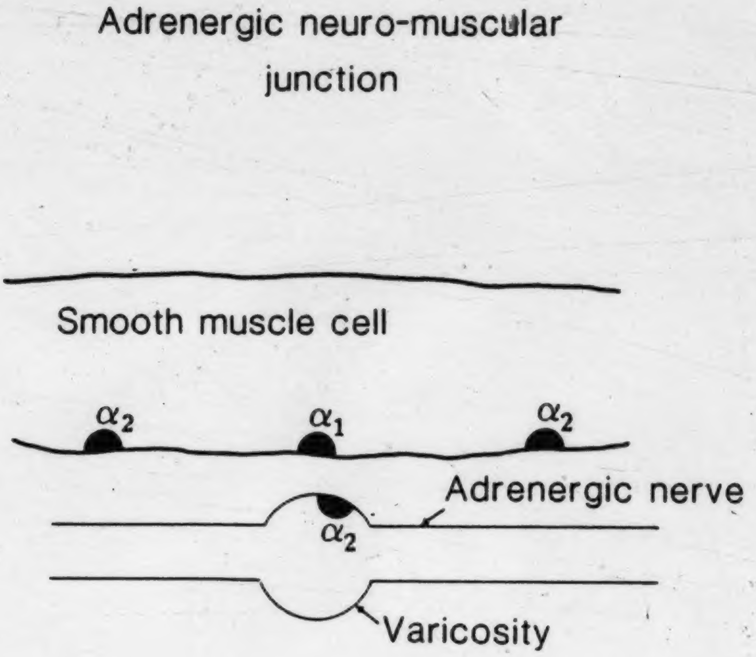

Adrenergic neuro-muscular junction
Smooth muscle cell
α_2
α_1
α_2
Adrenergic nerve
α_2
Varicosity

Figure 1. A diagrammatic representation of the postulated locations for α-adrenoceptor sub-types in adrenergically innervated smooth muscle. Pre-junctional α-receptors located on the axonal membrane are believed to be α_2 in type. Post-junctional α-receptors are of two types (1) α_1-receptors believed to be located at the synaptic cleft i.e., intrajunctionally. (2) α_2-receptors: believed to be located away from the synaptic cleft, i.e., extrajunctionally.

whole range of the dose-response and stimulus-response curves. This permitted any progression of the interaction to be studied. Canine lateral saphenous veins were selected for the present investigation for the following reasons:

1. the canine saphenous vein has been shown to possess a good sympathetic innervation with nerves penetrating almost up to the tunica intima: refer to literature review for details(17,18).
2. the presence of post-synaptic α_1 and α_2 receptors have been demonstrated in the isolated canine saphenous vein(12).
3. the isolated saphenous vein responds well to both exogenous noradrenaline and TNS.

LITERATURE REVIEW

The vascular system can be divided into the arteries, the veins and the microvasculature(19,20)(Fig. 2). The arteries are generally subdivided into two categories, the elastic arteries and the muscular arteries. Elastic arteries contain many elastic laminae in their walls and generally have large luminal diameters (e.g. aorta, carotid arteries). Potential energy, stored during cardiac contraction in the elastic tissue of the aorta and its branches is reconverted into kinetic energy for the circulation during the diastolic phase. This elastic recoil of the vessels sustain the pressure head better and renders blood flow to the periphery steadier than it would otherwise be. Muscular arteries which are formed by branching of elastic arteries have less elastic tissue and more smooth muscle cells in their walls. Collectively, the arterioles, the pre-capillary sphincters, the capillaries and the post-capillary venules are referred to as the microvascular bed. The arterioles which are 0.1-0.8 mm in diameter in the dog(19), have the smallest lumen: wall ratio (approximately 0.4) among all blood vessels and thus contribute most to the resistance to blood flow in the vascular system. These pre-capillary resistance vessels (arterioles) usually exhibit an efficient local myogenic control of their own vascular radius, and on this myogenic tone is superimposed an extrinsic neural control effected by autonomic nerves. The pre-capillary sphincters, themselves part of the pre-capillary resistance vessels, are particulary important in determining the size of the capillary exchange which is perfused at any moment in the tissue: an increase in the potency of the sphincters causing an increase in the number of capillaries open. The capillaries function as exchange

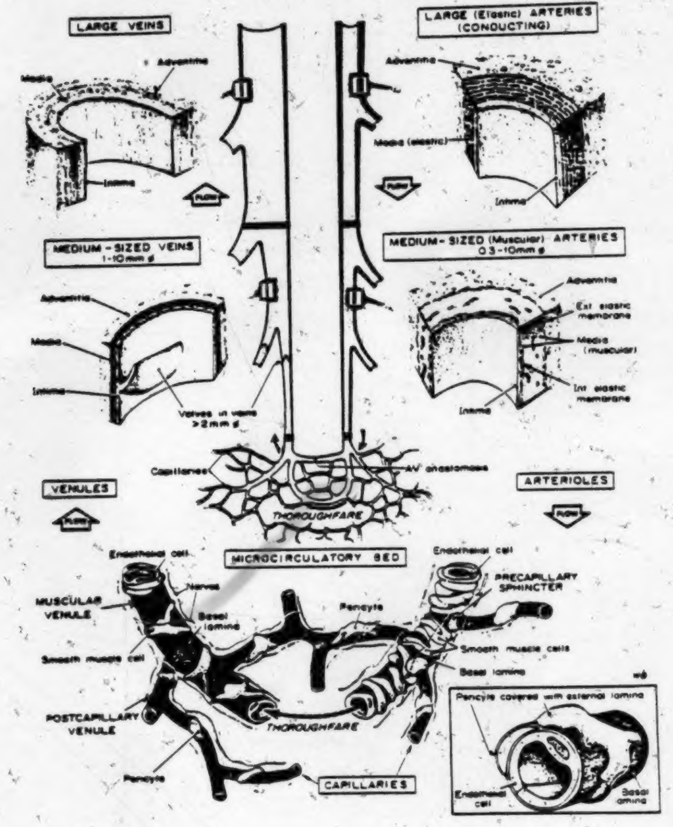

LARGE VEINS
Adventitia
Media
Intima
FLOW
LARGE (Elastic) ARTERIES (CONDUCTING)
Adventitia
Media (elastic)
Intima
FLOW
MEDIUM-SIZED VEINS 1-10mm ø
Adventitia
Media
Intima
Valves in veins >2mm ø
MEDIUM-SIZED (Muscular) ARTERIES 0.3-10mm ø
Adventitia
Ext elastic membrane
Media (muscular)
Int elastic membrane
Intima
Capillaries
AV anastomoses
VENULES
FLOW
ARTERIOLES
FLOW
THOROUGHFARE
MICROCIRCULATORY BED
Endothelial cell
Nerves
MUSCULAR VENULE
Basal lamina
Smooth muscle cell
POSTCAPILLARY VENULE
Pericyte
Endothelial cell
PRECAPILLARY SPHINCTER
Pericyte
Smooth muscle cells
Basal lamina
THOROUGHFARE
CAPILLARIES
Pericyte covered with external lamina
Endothelial cell
Basal lamina

Figure 2. Schematic diagram summarising the major structural characteristics of principal segments of blood vessels in mammals [Reproduced with permission; from Rhodin JAG, Handbook of Physiology(20)].

vessels where exchange of nutrients and metabolic waste products between the blood and the tissues take place. The veins are generally divided into large and medium-sized veins. They tend to have the greatest lumen: wall ratio among blood vessels and these offer little or no resistance to the flow of blood. They also possess valves to prevent back-flow of blood within the venous system. The veins or capacity vessels are important sites of change in the capacity of the vascular system. Changes in luminal configuration (from elliptical to circular cross-sectional profiles) and changes in myogenic tone of the veins induced by sympathetic constrictor nerves are of great importance in adjusting the capacity of the vascular system, particularly in postural changes.

MORPHOLOGY OF THE VESSEL WALL(20)

The walls of blood vessels (with the exception of the capillaries) consists of three layers: tunica intima, tunica media, tunica adventitia (Fig. 2, Fig 3). The tunica intima, the innermost layer of the vascular wall is composed of the following structures:

1. a single layer of endothelial cells lining all blood vessels
2. a basement membrane 80 nm in thickness
3. the sub-endothelial layer - composed of collagen fibres, elastic fibrils and smooth muscle cells; this sub-endothelial layer is usually present only in the large elastic arteries.

The endothelial cells are flat and elongated with their long axes parallel to that of the blood vessels. They are approximately 10-20 μm in length and 5 μm in width at their widest point. They have a thickness of about 0.2-0.5 μm with a slight bulge at the region of the nucleus. Their morphology can be best studied by pressure-perfusion

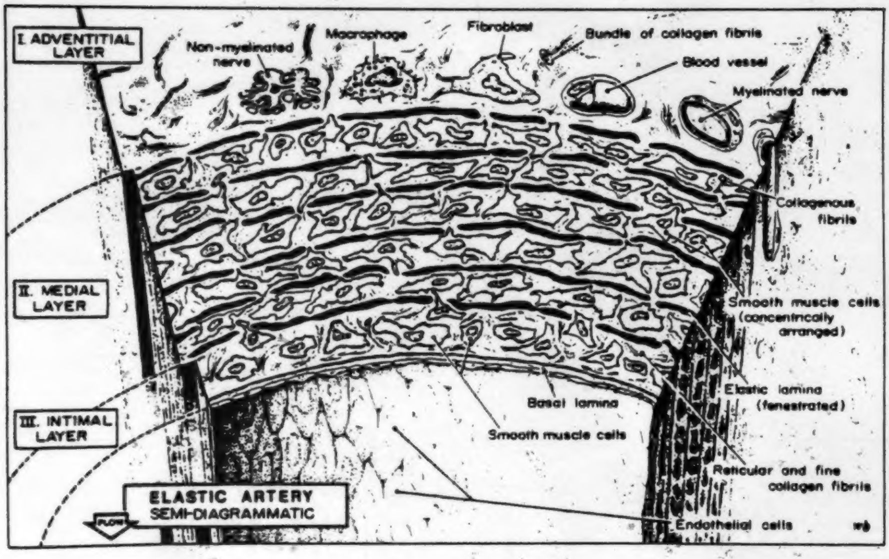

I. ADVENTITIAL LAYER
Non-myelinated nerve
Macrophage
Fibroblast
Bundle of collagen fibrils
Blood vessel
Myelinated nerve
Collagenous fibrils
II. MEDIAL LAYER
Smooth muscle cells (concentrically arranged)
Elastic lamina (fenestrated)
Basal lamina
III. INTIMAL LAYER
Smooth muscle cells
Reticular and fine collagen fibrils
ELASTIC ARTERY SEMI-DIAGRAMMATIC
FLOW
Endothelial cells
wb

Figure 3. A diagrammatic representation of the wall of an elastic artery with well organised elastic laminae in the medial layer (tunica media) [Reproduced with permission; from Rhodin JAG, Handbook of Physiology(20)].

with the fixative at a pressure equal to the normal intravascular pressure in the vessel studied. Collapse and wrinkling of the endothelial layer takes places unless pressure-perfusion is used during fixation. The endothelial cells are bound to each other by tight junctions(zona occludentes) and communicating junctions(gap junctions; maculae communicantes). The tight junctions are areas where the opposing endothelial cell membranes have fused along ridge-like protrusions of the individual cells. In section, these appear as punctate fusions of the two cell membranes. The gap junctions are patches (maculae) of opposing cell membranes consisting of a polygonal lattice of cell membrane sub-units. They function as sites for cell-to-cell transfer of ions and metabolites (ionic, electrotonic and metabolic couplings). Both these types of junctions are more common in arterial than venous endothelial cells.

The sub-endothelial layer, as stated before, is present only in the large elastic arteries. In the human aorta it undergoes a series of changes during life. At birth it is a thin structure with a narrow layer of connective tissue fibres. In young adults it increases in thickness and becomes fibrous and cellular by middle age. In senile subjects the layer is thick, fibrous and hyalinised. In some species (e.g. pig, man) smooth muscle cells are present in the sub-endothelial layer of the aorta. In small arteries and arterioles (where the sub-endothelial layer is lacking), endothelial cells, and smooth muscle cells in the media are closely connected through myo-endothelial junctions. These are cytoplasmic processes from the endothelial cells or the smooth muscle cells and they penetrate the basal lamina and if present, the internal elastic lamina. There are few of these processes in small arteries but they become increasingly numerous as the arterioles approach their terminal ramifications and the pre-capillary sphincters.

The tunica media is the middle layer of the vascular wall. Smooth muscle cells are the principle constituent of the media but it also contains a varied number of elastic laminae, collagen fibrils and elastic fibrils(Fig. 3, Fig. 4). It is usually bounded by the internal and external elastic laminae and is thicker in the arteries than in the veins. The tunica media of the human aorta contains about 40-60 fenestrated elastic laminae. These elastic laminae which are about 3 μm in thickness are concentrically arranged and spaced equidistantly. They are interconnected by a network of elastic fibrils and this highly structured and elaborate elastic framework gives the media its great resilience and strength in the elastic arteries. The smooth muscle cells are found within this framework. The elastic laminae diminish in number and in organisation, the smaller the size of the artery with arterioles generally lacking elastic laminae. Most of the smooth muscle cells in the media are oriented obliquely running diagonally at small angles between the elastic laminae.

The smooth muscle cells in the media tend to form a spiral although the orientation may vary with the distending forces(21). Wolinsky and Glagov(21) in a study of the rabbit aorta fixed below diastolic pressure, showed that the smooth muscle cells are oriented obliquely or even perpendicularly with respect to the elastic laminae. However, if the preparations were fixed at pressures equal to or greater than the diastolic pressure, the smooth muscle cells appeared to be elongated and arranged helically between the elastic laminae. Furthermore, the pitch

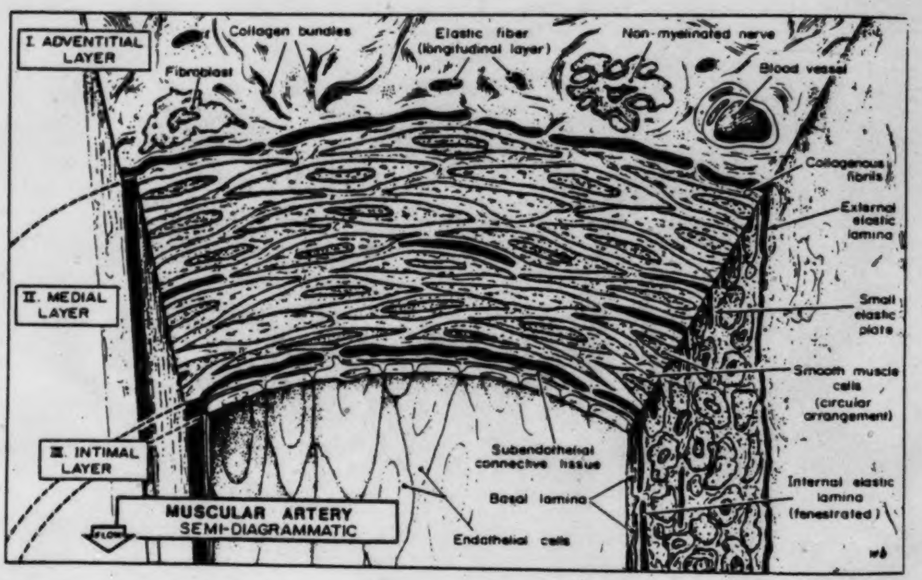

I. ADVENTITIAL LAYER
Collagen bundles
Elastic fiber (longitudinal layer)
Non-myelinated nerve
Fibroblast
Blood vessel
Collagenous fibrils
External elastic lamina
II. MEDIAL LAYER
Small elastic plate
Smooth muscle cells (circular arrangement)
III. INTIMAL LAYER
Subendothelial connective tissue
Basal lamina
Internal elastic lamina (fenestrated)
MUSCULAR ARTERY SEMI-DIAGRAMMATIC
FLOW
Endothelial cells
wb

Figure 4. A diagrammatic representation of the wall of a muscular artery. [Reproduced with permission, from Rhodin JAG, Handbook of Physiology(20)].

of the helix can vary depending on the contractile status of the vascular wall. It is conceivable that in a muscle spiral with extensive mobility, the pitch of the helix may change considerably and the direction of the muscle cells may alter from longitudinal to diagnonal to perpendicular, or to ring shaped depending on the functional state of the blood vessel.

This, apparent helical arrangement of the smooth muscle cells in the media is the basis for the helically cut strip first introduced by Furchgott and Bhadrakom in 1953(22). The helical cutting would yield strips that possess muscle cells which were oriented more or less parallel to the long axis of the strip. However, as the orientation of the smooth muscle cells varies in different blood vessels (even in the resting state), the angle of cut during the preparation of the strip would determine the amount of stress that could be developed by the strip(23).

Muscular arteries, as their name implies, are well endowed with smooth muscle bundles in their media(Fig. 4). However, with further diminution of the size of the arteries, the amount of smooth muscle cells in their media diminish, with arterioles by definition having only 2-3 layers of smooth muscle cells. Electron microscopic studies by Rhodin, of the small dermal arteries of the rabbit, demonstrated that the innermost layers of smooth muscle cells were arranged transverse to the long axis of the vessel, whereas the outermost layers were arranged spirally with a pitch of 18°(24). The media of venules and small veins contain a small number of smooth muscle cells separated by abundant collagen fibrils. Although a few elastic fibrils are present, no distinct elastic laminae are present in these vessels. In the walls of some medium and large sized veins, smooth muscle cells are present in abundance although these cells are often located outside the zone that is classically regarded as the media (i.e. in the sub-endothelial tissues and in the tunica adventitia). However, as the external and internal elastic laminae are not present in veins, the distinction between the intimal, medial and adventitial layers is not as clear as in arteries. The smooth muscle cells in veins are arranged longitudinally and circularly as well as helically in different types of veins. Thus, the rat portal vein contains two distinct layers of smooth muscle cells, an inner, narrow sub-endothelial layer with circularly oriented cells, and an outer, wider layer with longitudinally arranged smooth muscle cells(25). In the bovine mesenteric vein the contractile response of a longitudinally cut strip to transmural nerve stimulation was about 20 times that of a circularly cut strip indicating the predominance of longitudinally arranged smooth muscle cells in these veins(26). The walls of veins contain abundant collagen fibres separating individual smooth muscle cells as well as layers of smooth muscle.

Tunica adventitia is the outermost layer of the vascular wall. The thickness of this layer varies considerably depending on the type and location of the blood vessel. For instance, the cerebral blood vessels almost lack an adventitia. The adventitia consists of dense fibro-elastic tissue and the nutrient vessels of the blood vessel wall (arterioles, capillaries, venules and lymphatic tissue, together referred to as the vaso-vasorum). There is no smooth muscle cells in the adventitia except in large veins which contain longitudinally arranged smooth muscle cells. The adventitia also contains nerves. These nerves are of two types: 1) part of the peripheral nerve trunks for sensory organs, muscles and visceral organs 2) vasomotor and sensory nerves for the vascular wall itself. The adventitia functions to anchor the blood vessel to its surrounding tissues by way of loose connective tissue. In the elastic arteries, the adventitia tends to be thin especially in the larger vessels and contains a loose network of elastic fibrils. The tunica adventitia tends to be wider in the muscular arteries often occupying half of the vessel wall. The adventitial layer is thin and inconspicuous in arterioles and venules merging with the surrounding connective tissue. This layer is best developed in medium-sized and large veins comprising up to 75 per cent of the vascular wall. Here the adventitia is made up of collagen bundles, elastic fibres and smooth muscle cells. The collagen bundles are arranged in a helical fashion forming an intimate relationship with the smooth muscle cells in the adventitia. This relationship and the firm attachement of the adventitia to the surrounding connective tissue allow the adventitial layer to play a more direct role in vascular dilatation in veins as compared with the arteries.

Capillaries comprise a dense network of narrow (3 μm radius) short (750 μm) tubes with total wall surface areas which are enormous in relation to the length of the constituent tubes. The total capillary exchange surface area in a man of 70 kg body weight, is believed to be 550-600 square meters. This calculation,, however, assumes a situation in which all systemic capillaries are patent which is almost never seen in life.. At rest, approximately 25 per cent of the total number of capillaries are patent, thus giving a total cross sectional area of 150-200 square meters(27). Capillaries lack both a media and an adventitia. However, a cell type known as the pericyte is intimately associated with these vessels: the pericyte is neither a smooth muscle cell nor a fibroblast. Structurally, it is surrounded by an external lamina, and it contains a small number of micro-filaments in its cytoplasm. It could represent a potential smooth muscle cell or function as a phagocytic cell.

Morphology of the canine lateral saphenous vein

Most of the studies on the histology of this vein has been done by Osswald & Guimaraes(17). This is a medium sized vein 3-5 mm in diameter containing an abundance of smooth muscle fibres. The tunica intima in this vein is made up of a single layer of endothelial cells and the basement membrane, with no definite sub endothelial layer. The tunica media, having a thin, discontinuous elastic lamina as its innermost layer is relatively thick containing 8-12 layers of smooth muscle cells. These smooth muscle cells are mostly circularly oriented although there are longitudinal as well as oblique fibres. This predominantly circular arrangement of the smooth muscle cells accounts for the very much greater tension developed by ring preparations of the vessel compared with helically cut strips. The smooth muscle fibres of the vein wall were found to make contact with each other at their ends and also laterally although spaces of variable width containing collagen and tenuous elastic fibrils were often found between the muscle cells in the above studies. The tunica adventitia in the saphenous vein is well developed with numerous elastic fibres.

ULTRA STRUCTURE OF SMOOTH MUSCLE CELLS(28,29,30,31,32)

Smooth muscle cells have an elongated but irregular shape with multiple cellular protrusions. They are a 40-100 μm in length and 2-5 μm in width, thus much smaller compared with cardiac muscle cells which are 10-20 μm in diameter but approximately of the same length. The smooth muscle cells have a single nucleus situated in the middle at the widest portion of the cell body. Each cell is surrounded by its plasma membrane (80 A in thickness) and there is no protoplasmic continuity between cells. The plasma membrane and the basement membrane, when the latter is present, constitute the sarcolemma. The presence of a distinct basement membrane is regarded as being one of the ultrastructural diagnostic features of smooth muscle, and it has been demonstrated in a variety of blood vessels. In tight junctions between smooth muscle cells, the fused plasma membranes of adjacent cells are devoid of basement membranes(31). The small intercellular space between smooth muscle cells contains blood vessels, nerve fibres, extracellular matrix and reticular fibres. Connective tissue cells are rarely found in the interstices of the sheets of smooth muscle. These muscles do not possess a distinctive tissue "capsule" or perimysium characteristic of skeletal muscle fibre(33).

The plasma membrane of smooth muscle cells contains multiple small flask shaped invaginations termed caveolae or surface vesicles. These are 50-80 nm in diameter and are also present in endothelial cells and fibroblasts. The extracellular space continues into these caveolae as demonstrated by the fact that extracellular markers such as colloidal Lanthanum (La^{3+}) and tannic acid enter these vesicles(32,34). These are not randomly scattered over the cell surface but tend to have a longitudinal orientation in smooth muscle(32). These caveolae increase the surface membrane area per cell volume by 25-70 per cent. Nevertheless, they are far less extensive compared to the transverse (T) tubular system in skeletal and cardiac muscle. Recent observations in the mouse coronary artery indicated that these surface vesicles may occur singly or in more complex forms that resemble chains of beads fused end to end. These beaded tubules may be composed of as many as 17 vesicles and they often extend deep into the cells(35). Smooth muscle lacks T-tubules and some investigators believe that caveolae are analagous to the T-tubules although no conclusive evidence is available at present. The surface vesicles may not be fixed structures and their distribution may reflect the functional status of smooth muscle(36). Occasionally in electron micrographs, some of the surface vesicles appear to be intracellular and have therefore been described as pinocytotic. However, the penetration of extracellular markers into these vesicles after fixation and the examination of tilt pairs of micrographs indicate their true extracellular nature(32). This artifactual intracellular appearance is probably due to their narrow necks which may not "enter" into the plane of section. Nevertheless, the possibility that they may become pinocytotic under some conditions cannot be definitely ruled out. Both mitochondria and sarcoplasmic reticulum have been observed to be closely associated with the surface vesicles, with the intervening distance being as small as 3-6 nm in some cases(34). However, the association of the sarcoplasmic reticulum with these surface vesicles is far less common compared with the association between the sarcoplasmic reticulum and T-tubules in skeletal muscle.

The sarcoplasmic reticulum in smooth muscle, as in striated muscle can be divided into the rough endoplasmic reticulum, with its associated ribosomes and the smooth endoplasmic reticulum. These are continuous with one another and also with the nuclear membrane, but not with the lumen of the golgi apparatus. Extracellular markers such as colloidal lanthanum, horse radish peroxidase and ferritin do not enter the sarcoplasmic reticulum confirming their true intracellular nature(34). The sarcoplasmic reticulum of smooth muscle is not as well organised as in striated muscle. However, the tubules of the reticulum are distributed throughout the cell and they approach the sarcolemma forming surface couplings. The two membranes may be as close as 10-12 nm at these surface couplings and this intervening space has been observed to be traversed by electron-dense bridging structures which have a periodicity of 20-25nm (34,37). The sarcoplasmic reticulum also forms close couplings with the surface vesicles(29). It is thought that the twitch contractions of vascular smooth muscle, triggered by action potentials are mediated via the release of calcium from sarcoplasmic reticulum at these couplings(38) but no conclusive evidence is available at present. The volume of the sarcoplasmic reticulum shows a significant variation between different types of smooth muscle(39). In the rabbit portal-anterior mesenteric vein and the taenia coli it constitutes only 2 per cent of the cytoplasmic volume. On the other hand, in the rabbit main pulmonary artery and aorta it amounts to 5.0-7.5 per cent of the total cell volume(33,38). There is a direct correlation between the volume of the sarcoplasmic reticulum and the ability of smooth muscles to contract in calcium-free solutions(32,33). The sarcoplasmic reticulum is regarded as a major calcium source and sink (i.e. a sequestration-site) in smooth muscle. Strontium has been used to demonstrate the ability of the reticulum to accumulate divalent cations as it is more electron dense than calcium due to its higher atomic number(37). The electron-dense deposits can be identified as strontium by electron probe analysis.

The mitochondria in smooth muscle were previously considered as a possible source and sequestration site for calcium in smooth muscle. They too are often closely associated with the surface vesicles, a 4-5 nm distance separating the two membranes. Recent studies, however, fail to show any evidence for a role for mitochondria in the physiological (as opposed to pathological) regulation of cytoplasmic calcium. In the presence of Mg^{2+}, isolated vascular smooth muscle mitochondria(39,40) have a rather low affinity for Ca^{2+} (apparent Km = 17 μmol), that is inconsistent with the requirements of a physiological relaxing system. However, in damaged muscle fibres, massive mitochondrial calcification in the form of granules has been readily demonstrated(41). In these cells intracellular Na^+ and Ca^{2+} were high and the intracellular K^+ low, suggesting that mitochondrial calcium loading may have been due to the abnormally high cytoplasmic Ca^{2+} caused by cell damage.

Smooth muscle cells contain an elongated ellipsoidal nucleus containing one or two nucleoli and a double nuclear envelope in the relaxed cells. However, the nucleus becomes highly compressed and convoluted in contracted cells(36).

A variety of cell junctions are present between smooth muscle cells. These structures, are believed to play a role in ionic and metabolic cell-to-cell communication and are also present in other excitable cells such as cardiac and nerve cells and in non-excitable cells such as endothelial cells, liver parenchymal cells and fibroblasts(32). Gap junctions or nexuses having a 2-4 nm gap between the outer leaflets of the opposed cell membranes can occur between two parallel cell membranes, between opposed cell protrusions and between invaginations of one cell into its neighbour. The entire width of the junction (including gap and membranes) is 15-19 nm. Extra-cellular markers such as horseradish peroxidase and colloidal lanthanum penetrate the gaps in these junctions. The second type of junction that is believed to be present in smooth muscle cells is the tight junction or zona occludens which is not penetrated by these extracellular markers. Freeze fracture studies of the tight junctions show a strikingly different appearance from gap junctions. They show a meshwork of ridges with furrows on the complementary face as compared with the 8.5 nm diameter particles and corresponding pits seen in freeze fractured gap junctions(32). Other types of junctions such as intermediate contacts (attachment plaques), septate junctions and simple appositions are also found in smooth muscle. In electrically excitable cells, gap junctions provide a low-resistance pathway for the spread of depolarization throughout the tissue. In smooth muscle, although all cells appear to be electrically coupled to one another, the extent of this coupling varies widely from tissue to tissue(42,43). The structure responsible for this coupling is also not established but the gap junctions are favoured by many workers as the evidence for their involvement in ionic coupling in other tissues is very strong(42,43). The gap junctions are also believed to participate in metabolic coupling allowing transfer of small molecules such as amino acids, sugars and nucleotides from cell to cell.

Ultrastructure of the canine lateral saphenous vein

Electron microscopic study of the tunica media of this vein(18) demonstrated that the numerous smooth muscle cells present contained many short projections and indentations on their surfaces. The projections from adjacent cells were often opposed forming cell junctions. At these junctions the intervening space was approximately 20 nm in width and the opposing sarcolemmal membranes often demonstrated an electron density. The cells contained many oval shaped surface vesicles 80 nm in width and 90 nm in length. The interstitial space between the smooth muscle cells contained a variable amount of collagen fibrils, an amorphous filamentous background rich in 10 nm thick microfibrils and rare elongated fibrocytes.

STRUCTURE AND CHEMISTRY OF THE CONTRACTILE PROTEINS

As in skeletal and cardiac muscle a sliding-filament mechanism is believed to operate in smooth muscle contraction. The elements of the contractile apparatus include three types of filaments (thick, thin and intermediate), dense bodies and attachment plaques. When isolated smooth muscle is observed to contract, small closely spaced blebs form on the cell surface interspersed with undistorted regions(44). It is believed that the latter are sites of attachments of the contractile elements to the sarcolemma and have been named attachment plaques. Dense bodies, too, are similar structures but dispersed throughout the sarcoplasm and believed to be intracellular attachment sites for the contractile elements analogous to the Z-lines in cardiac muscle. α-actinin similar to that found in skeletal muscle has been isolated from some types of smooth muscle. In skeletal muscle α-actinin is believed to form part of the Z-line structure. In smooth muscle α-actinin has been localised in the dense bodies (using an antibody technique).

Smooth muscle cells contain 3 types of filaments in their contractile apparatus: the thick filaments (13-20 nm in diameter), the thin filaments (5-8 nm in diameter) and the intermediate filaments (approximately 10 nm in diameter). The force generating apparatus in smooth muscle is organised less intricately than in cardiac muscle, in that highly structured parallel arrays of interdigitating filaments characteristic of cardiac muscle are not seen(45). Rather, one finds throughout the cytoplasm, large numbers of filaments arranged in directions roughly parallel to the longitudinal axis of the cell. When the muscle is relaxed, the filaments tend to lie parallel to the long-axis of the smooth muscle cells. However, when the muscle cells contract, the filaments tend to assume a progressively oblique direction to the long axis. At maximal contraction the angle between the long axis of the cells and the filaments may be as much as 25-40°(46).

Thick filaments

The thick filaments are fibrous(α-helix) in structure and contain protrusions with globular heads along their length (Fig. 5). They are comprised of upwards of 200 myosin molecules (relative molecular mass:470,000). The myosin molecule can be enzymatically cleaved into two parts(29).

1. Light meromyosin (LMM, relative molecular mass: 150,000) - this is a fibrous protein which forms the "tail" of the myosin molecule (150 ± 20 nm in length) and lines up in a sheath with other similar molecules to form a thick filament.
2. Heavy meromyosin (HMM) - this appears as protrusions from the thick filaments. These protrusions form the cross-bridges between the thick and thin filaments during contraction. The heavy meromyosin can be further sub-fractionated into a fibrous S-2 segment (relative molecular mass: 60,000) and a globular S-1 segment containing the two heads of the myosin molecule (2 x 120,000). The S-1 segment is composed of two identical units,

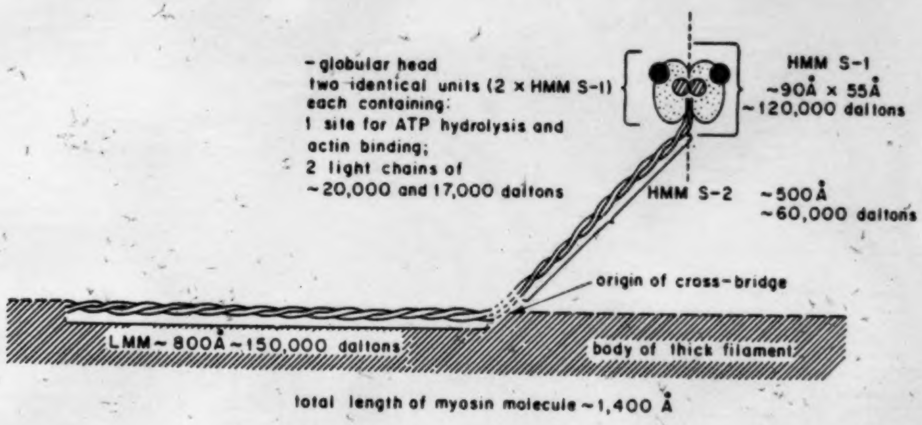

- globular head
two identical units (2 × HMM S-1)
each containing:
1 site for ATP hydrolysis and
actin binding;
2 light chains of
~20,000 and 17,000 daltons
HMM S-1
~90Å × 55Å
~120,000 daltons
HMM S-2
~500Å
~60,000 daltons
origin of cross-bridge
LMM ~ 800Å ~ 150,000 daltons
body of thick filament
total length of myosin molecule ~ 1,400 Å

Figure 5. Diagrammatic representation of the myosin molecule composed of the light meromyosin (LMM), heavy meromyosin subfragment-1 (HMM S-1) and heavy meromyosin sub fragment-2 (HMM S-2). The α-helical portion of the molecule is depicted as a rope-like structure. At the origin of the cross-bridge, i.e., one end of the HMM S-2 molecule, it is not known whether the α-helical structure is retained (dashed line). The position of the four light chains (shown as the smaller circles in the globular head) is completely arbitrary. Conformation of the globular head differs from the rest of the molecule in that it is not predominantly an α-helix, and this difference is presented diagrammatically. [Reproduced with permission, from Hartshorne DJ and Gorecka A, Handbook of Physiology(53)].

each containing an attachment site for actin, an enzymatic site that hydrolyses adenosine triphosphate (ATP) to liberate energy used up for the contraction, and two "myosin light chains" that are involved in the function of these segments. Although striated muscle also contains myosin light chains, there are distinct differences in structure and function from those of smooth muscle. The light chain composition is to a degree characteristic of a particular myosin. Smooth muscle myosin contains two light chains of relative molecular mass 20,000 and two light chains of relative molecular mass 17,000 (one of each in the two heads): myosin from cardiac muscle has two classes of light chains of relative molecular mass 27,000 and 20,000(28). In general, myosin from non-muscle sources resembles smooth muscle type in its light chain composition.

The heavy meromyosin and the light meromyosin appear to form a flexible "hinge" between them. The fibrous heavy meromyosin functions to transmit the force generated by the operational site of the myosin molecule, to the light meromyosin. Myosin molecules are assembled into filaments as a tail-to-tail biopolar segment at the centre, and extends in a head-to-tail fashion on either side of the centre in striated muscle(32). Thus the arrangement of the myosin molecules in one half of the striated muscle cells is opposite to that of the other half (bi-polar arrangement). In smooth muscle a central bare zone has not be unequivocally demonstrated in situ, nor have M-protein and M-bridges that form the M-line in the centre of the A band in skeletal muscle been identified. The presence of a central bare-zone suggests a bi-polar arrangement as described above. Some workers have suggested that in smooth muscle, myosin molecules are packed so that their heads are oriented in only one direction: i.e. each half or face of the filament has the same polarity (unipolar arrangement)(28,47).

Thin filaments

The thin filaments contain the contractile proteins actin and tropomyosin in smooth muscle. Troponin present in striated muscle as a protein which regulates contractile activity, is absent in smooth muscle. Actin, the major component of the thin filament, is present in all muscle cells and in almost all eukaryotic cells. It is therefore not surprising that there appears to be little difference in the chemical composition of actin from different sources. The thin filament is composed of a double-stranded helical filament (Filamentous/Fibrous actin, F-actin) with an axial repeat of about 36-38 nm(32). The F-actin is a polymer of the (globular) G-actin molecules (5.5 nm in diameter). The G-actin molecule is a single chain protein with a relative molecular mass of 42,000. The S-1 subfragment of the heavy meromyosin (i.e. the globular heads of the myosin molecule) binds to the thin filaments to form cross-bridges. Two functional roles suggested for the actin in the contractile process are: (1) the transmission of force delivered by the movement of the cross bridge of the myosin molecule (2) activation of the enzyme actomyosin-ATPase to release free energy for contraction(29) (See under Contractile Mechanisms in Smooth Muscle).

Tropomyosin, like actin, is similar in structure in all types of smooth muscle. Although minor variations in sub-unit composition are present among tropomyosins from different sources, these are not sufficient to indicate any major alterations in their properties. The important property retained by all tropomyosins is the ability to bind to F-actin(48). Tropomyosin forms an alpha-helix lying in the two grooves of the double-strand of F-actin and is composed of tropomyosin molecules (relative molecular mass: approximately 70,000). The molar stoichiometry of actin to tropomyosin (approximately 7:1) is similar in all types of muscle. Because of the absence of troponin, the function of tropomyosin in smooth muscle is not clear at present.

In general, smooth muscle contains less myosin than skeletal muscles. The myosin content in smooth muscle is believed to be about 20 mg per g cell wet weight compared with 62 mg per g cell wet weight for skeletal muscle(28). Although smooth muscle has a 3-5 fold lower concentration of myosin compared to skeletal muscle, its actin content is higher than in skeletal muscle. Thus the actin: myosin ratio is much higher in smooth muscle (molar ratio 17-29 compared to 4 in skeletal muscle). In spite of this lower content of myosin the maximum force developed by smooth muscle is equal to or greater than that developed by striated muscle. Hartshorne(28) and Murphy(49) have discussed the possible explanations for this apparently anomalous finding. These explanations are listed below. However, there is no conclusive evidence for any of them at present.

1. The greater length of the thick filaments in smooth muscle (2.2 μm compared with 1.5-1.6 μm in vertebrate striated muscle) and the larger thin-to-thick filament ratio could result in simultaneous engagement of a larger number of cross-bridges.
2. Different thick filament packing mode: the connective tissue may be arranged to couple more cells in parallel than are anatomically present in a cross section of the tissue leading to a mechanical advantage. The high force generating capacity of

smooth muscle could result from such an arrangement of cells whose individual ability to develop force is more in keeping with their myosin content(48). The oblique arrangement of the filaments during contraction in smooth muscle may also offer a mechanical advantage as suggested by Rosenbluth(50).

3. A longer contact time of the cross-bridge with actin during the cross-bridge cycle.

Intermediate filaments

The intermediate filaments are the third type of filamentous structure found in smooth muscle, and have a diameter of approximately 10 nm. These filaments are generally associated with dense bodies, often surrounding them. In transverse section they appear hollow. Similar filaments have been observed in a variety of non-muscle cells, e.g. glial filaments, filaments of fibroblasts and endothelial cells. The intermediate filaments are not attached directly to either thick or thin filaments and do not appear to play a direct role in the contractile process. They are dispersed throughout the sarcoplasm and are believed to play a role in the cell architecture as a cytoskeleton by forming bridges across dense bodies. The intermediate filaments are made up of a protein named skeletin (relative molecular mass: 55,000). Their number may increase in abnormal smooth muscle fibres and crowd out thick and thin filaments(32). The intermediate filaments are believed to form a cystoskeleton but no definite evidence is available at present.

α-actinin similar to that found in skeletal muscle has been isolated from some types of smooth muscle. In skeletal muscle α-actinin is believed to form part of Z-line structure. In smooth muscle α-actinin has been localised in the dense bodies, using an antibody technique. The dense bodies form an anchor for the thin filaments in smooth muscle and are believed to serve as an equivalent to the Z-lines of striated muscle.

REGULATION OF CONTRACTION IN SMOOTH MUSCLE

In 1954, A.F. Huxley & Niedergerke(51) and H.E. Huxley & Hanson(52) proposed independently that the shortening of striated muscle was the result of a relative sliding between two sets of filaments. This method of shortening known as the sliding filament mechanism, firmly established in striated muscle, is also believed to operate in smooth muscle contraction. The two sets of filaments have been identified as the thick and thin filaments. Neither filament type alters in length during shortening of the muscle, the change in length of the muscle being achieved by varying amounts of overlap between the thick and thin filaments. The thick filaments are composed of myosin molecules arranged in a manner such that the enzymatically active portion of the molecule protrudes from the body of the filament. These protruding portions, known as the cross-bridges, bind to the actin of the thin filaments during contraction to form sites of tension development. This interaction between the actin and myosin is responsible for shortening and isometric tension development. As in skeletal muscle the energy source during shortening in smooth muscle is Mg^{2+}-adenosine triphosphate (Mg^{2+}-ATP). The Mg^{2+}-ATP is hydrolysed during muscle contraction by the enzyme Mg^{2+}-adenosine triphosphatase (Mg^{2+}-ATPase) which is located in the heavy meromyosin S-1 fraction (HMM S-1) of the myosin molecule. This enzyme also known as actomyosin-ATPase determines the shortening velocity of the muscle, the maximal shortening velocity having a direct correlation with the enzyme activity(29). Vascular smooth muscle has a shortening velocity which is very much less than in skeletal muscle suggesting a marked difference in the actomyosin-ATPase activities in the two muscle types. The velocity of contraction is believed to be a function of the cycling rate of the cross-bridge. A cross-bridge cycle consists of the attachment of the bridge to the actin molecule, the sliding of the two filaments, detachment of the cross-bridge and the re-attachment of the bridge at a different site on the thin filament. On the other hand, the force generating ability of smooth muscle is equal to or greater than that in striated muscle as explained under Structure and Chemistry of the Contractile Proteins. The force of contraction (as opposed to velocity of contraction) is believed to be a function of the number of simultaneously active cross-bridges between the thick and thin filaments, and the efficiency of the transduction of the force generated by a single cross-bridge to overall force generation by the muscle.

Apart from the lower magnitude of the Mg^{2+}-ATPase activity in smooth muscle there are other *in vitro* features of this enzyme which are different in smooth muscle as compared with striated muscle(53). The smooth muscle enzyme requires a higher concentration of Mg^{2+} to generate maximum enzyme activity. The reason for this is not yet clear. Another feature that is different is the dependence of the ATPase activity on the concentration of myosin. At low concentrations of the enzyme the rate of product formation follows a linear dependence on the concentration of the enzyme in skeletal muscle. In smooth muscle this dependence is not linear. This effect may be related to the solubility of the actomyosin. At low concentrations of actomyosin the proteins are soluble and the ATPase activity is low; as more actomyosin is added the protein forms a precipitate that has a higher ATPase activity. Finally the Mg^{2+}-ATPase activity of smooth muscle is less affected by an increase in ionic strength in the surrounding medium (e.g. addition of KCl) than that of skeletal muscle.

As in striated muscle, an increase in intracellular (sarcoplasmic) free ionic calcium (Ca^{2+}) leads to contraction of the muscle cells and a decrease to relaxation. The increase in intracellular Ca^{2+} is regarded as the final common step in initiating contraction. Furthermore, the ionic concentration of Ca^{2+} required for activation are comparable in the two muscle types with about half-maximal activity at a Ca^{2+} concentration of about 10^{-6} mol/l.

Recently, free calcium indicators have been utilised to study the relationship between intracellular ionic Ca^{2+} concentration and the contractile state in smooth muscle cells(54,55). The bioluminescent photoprotein aequorin was used in these studies. Aequorin, first isolated in 1961 from the jellyfish aequorea forskalea (aequorea aequorea) is a calcium-activated photoprotein that emits light when it binds Ca^{2+} ions(56). Morgan and Morgan(55) using a microinjection technique to load vascular smooth muscle cells from Amphiuma tridactylum with aequorin demonstrated that contractions caused by exogenous drugs as well as electrical stimulation were associated with enhanced light responses which preceded the tension responses. The light response to electrical stimulation showed a transient peak which appeared to return to the basal level by the time the tension reached a peak. However, enhanced loading of the cells with aequorin (making the cells hyperpermeable to the compound) demonstrated a smaller, sustained increase in the light response following the initial transient peak.

The activator Ca^{2+} for contraction in smooth muscle comes from either intracellular or extracellular sources and these sources seem to differ between smooth muscle cells from different vessels and between different agonists used to initiate contraction. At present there is considerable controversy regarding the sources and sinks (i.e. sequestration sites) of Ca^{2+} in smooth muscle(30).

Although it is well established that increases in intracellular free Ca^{2+} leads to contraction of smooth muscle cells ultimately by activation of the actomyosin-ATPase, how Ca^{2+} effects this activation remains controversial. The molecular mechanisms by which Ca^{2+} regulates actin-myosin interaction have been examined in a variety of muscle types, e.g. vertebrate and invertebrate striated and smooth muscles, as well as in various non muscle motile systems e.g. platelets. Based on these studies a number of different regulatory mechanisms have been identified. These mechanisms can be divided into 3 groups:

1. myosin (or thick filament) linked regulation
2. actin (or thin filament) linked regulation
3. dual (or actin and myosin linked) regulation

Myosin-linked regulation

The myosin-linked regulatory system can be divided into two subtypes(29).

A. Regulation by myosin, with Ca^{2+} acting as a disinhibitor, e.g. molluscan smooth muscle(29). The regulatory system in this muscle lies in the myosin light chains (relative molecular mass: 20,000) which inhibit the activation by actin of the actomyosin-ATPase. This inhibition is removed in the presence of Ca^{2+} ions. However, if the myosin light chains are detached from the myosin molecules by treatment with ethyleneglycol-bis(β-aminoethyl ether) -N',N',N',N'-tetraacetic acid (EGTA, 10 mmol/l) the muscle cells can be activated by actin in the absence of Ca^{2+} ions. When the light chain from molluscan muscle or smooth muscle is added back to this system the Ca^{2+} requirement for activation is restored. Myosin light chains from mammalian skeletal or cardiac muscle cannot substitute for the former. Phosphorylation of the myosin light chains of molluscan muscle does not regulate the actomyosin-ATPase activity in this system.

B. Regulation by phosphorylation and dephosphorylation of the light chains of myosin, with Ca^{2+} acting as an activator of the phosphorylation reaction. The phosphorylation of the myosin light chain allows the activation by actin of actomyosin-ATPase leading to contraction. This theory known as the phosphorylation theory is believed to operate in vertebrate smooth muscle by the majority of investigators. The theory is discussed in detail below.

Actin-linked regulation

Three sub-types of actin-linked regulation have been identified(57)

A. Regulation by troponin is believed to be the primary regulatory system in vertebrate striated muscle. A mixture of pure actin and pure myosin exhibits a near maximal activity of actomyosin-ATPase (thus maximal contractile activity). Thus, Ca^{2+} is not necessary for the activation of the pure system. However, in intact resting muscle and in the extract of crude (native) actomyosin the Mg^{2+}-ATPase activity is inhibited by a complex cooperative influence of troponin and tropomyosin. This inhibitory action of troponin and tropomyosin is prevented by Ca^{2+} which therefore acts as a disinhibitor rather than a direct activator. Ebashi and his collaborators discovered this inhibitory effect of the troponin-tropomyosin molecules, which are bound to actin in the thin filament both *in situ* and when extracted in the native system(58). Thus, *in situ* and in the native system the formation of cross-bridges between actin and myosin are prevented by the presence of troponin and tropomyosin.

Striated muscle tropomyosin has a relative molecular mass of 66,000 and is composed of two helical sub-units that coil about each other. Tropomyosin molecules are polymerised end-to-end and form a strand that lies in the grooves of the actin filament (Fig. 6). The troponin in striated muscle exists as a globular complex of three proteins: troponin I which acts to inhibit the actomyosin-ATPase activity, troponin T which serves to bind the globular complex of troponin to tropomyosin, and troponin C which serves as a reversible binding site for Ca^{2+} ions.

In the absence of Ca^{2+}, troponin I binds tightly to actin and tropomyosin, maintaining tropomyosin in a position on the actin molecule that prevents the actin-myosin interaction(59). Binding of Ca^{2+} to troponin C results in a reduction in the binding affinity of troponin I to actin and tropomyosin leading to dissociation of troponin I from the latter. This in turn results in a shift in the position of tropomyosin in the actin groove. This new position no longer prevents the actin-myosin interaction leading to the formation of cross-bridges. During relaxation, Ca^{2+} is removed from the troponin C which ultimately

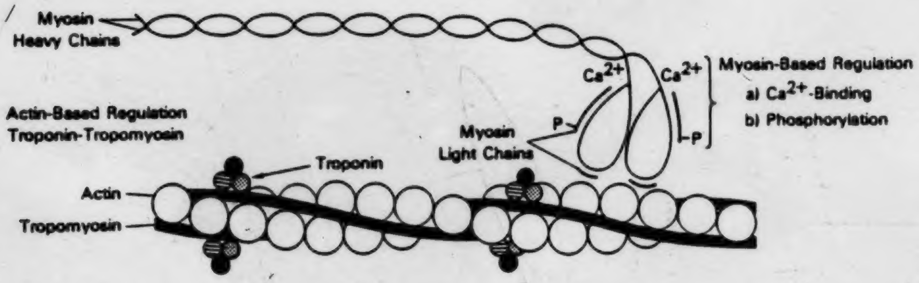

Myosin
Heavy Chains
Ca^{2+}
Ca^{2+}
Myosin-Based Regulation
a) Ca^{2+}-Binding
b) Phosphorylation
Actin-Based Regulation
Troponin-Tropomyosin
P
P
Myosin
Light Chains
Troponin
Actin
Tropomyosin

Figure 6. Diagrammatic representation of the structure of the thin and thick filaments and their interaction in muscle. The diagram shows components of three known regulatory systems for actomyosin-ATPase activation. (1) The thin filament regulatory system in which three troponin molecules (i.e. troponin-T, troponin-I and troponin-C) plus tropomyosin act cooperatively, in response to an increase in free ionised calcium to permit the activation of the actomyosin-ATPase of the globular portion of the myosin molecule by actin. (2) Actin activation of actomyosin-ATPase by a direct binding of calcium to myosin (3) The phosphorylation of the 20,000 dalton myosin light chain, thereby disinhibiting the actomyosin-ATPase. Not shown in this diagram is a second thin filament regulatory system that involves leiotonin molecules, which are bound to actin. Note: smooth muscle does not contain troponin [Reproduced with permission from the Annual review of Biochemistry, 1980; 49:925, Adelstein RS (Annual Reviews Inc.)].

results in a shift of the tropomyosin to the "old" position in the actin groove and thus an inhibition of the actomyosin-ATPase activity.

B. Regulation by leiotonin, a protein that is believed to be located in the thin filaments. This theory, known as the leiotonin theory, is an alternative to the phosphorylation theory (discussed above) in smooth muscle regulation. Leiotonin, in the presence of Ca^{2+}, activates the actomyosin-ATPase activity leading to contraction. Thus, leiotonin can be compared to troponin in striated muscle. However, unlike in striated muscle (which demonstrates near maximal Mg^{2+}-ATPase activity in a mixture of pure actin and pure myosin - without troponin - in the absence of Ca^{2+}) pure actin and pure myosin in smooth muscle does not have any ATP-ase activity in the presence of Ca^{2+} unless leiotonin and tropomyosin are present. This leiotonin theory is discussed in detail below.

C. Recent studies suggest the presence of a unique thin-filament linked regulatory system in certain smooth muscle types (e.g. pig aorta) that is characterised by phosphorylation of a basic protein (relative molecular mass: 21,000) in thin filaments(60). This phosphorylation of the thin filament protein results in an approximately four-fold increase in Ca^{2+} binding by the thin filaments. Further, the eight-fold decrease in the Ca^{2+} concentration which is required to switch on the thin filament activation of skeletal muscle actomyosin $Mg^{2\pm}$ATPase, was also produced by this phosphorylation.

Dual-linked regulation

Dual (actin and myosin-linked) regulation has been shown to occur in some invertebrate muscles(61). For instance, the skeletal muscle of Limulus (the horseshoe crab) has been shown to possess a thick filament-linked system (myosin phosphorylation) and a thin filament-linked system (troponin)(62).

In summary, the two most important theories are the phosphorylation theory and the leiotonin theory. These are discussed in detail below.

Phosphorylation theory

This, the more popular of the two theories, states that the key event in the activation of the Mg^{2+}-ATPase activity is the phosphorylation of the myosin light chains. The basic concepts behind this theory are(28,29,63) (Fig. 7):

1. In the relaxed muscle the sarcoplasmic Ca^{2+} is about 10^{-7} mol/l; myosin is in the non-phosphorylated state and cross-bridge interactions between actin and myosin are detached.
2. Contraction is initiated by an increase in sarcoplasmic free Ca^{2+} ions. This increased Ca^{2+} concentration activates the enzyme myosin light chain kinase (MLCK) which phosphorylates the two 20,000 relative molecular mass light chains of myosin. A maximum of 2 moles of phosphate can be incorporated per mole of myosin (1 mole for each light chain).
3. The light chain phosphorylation allows the activation by actin of Mg^{2+}-ATPase activity of myosin. This leads to cross-bridge formation and cross-bridge cycling between actin and myosin: it is assumed that as long as the Ca^{2+} concentration remains above the activation threshold, cross-bridge cycling will continue.

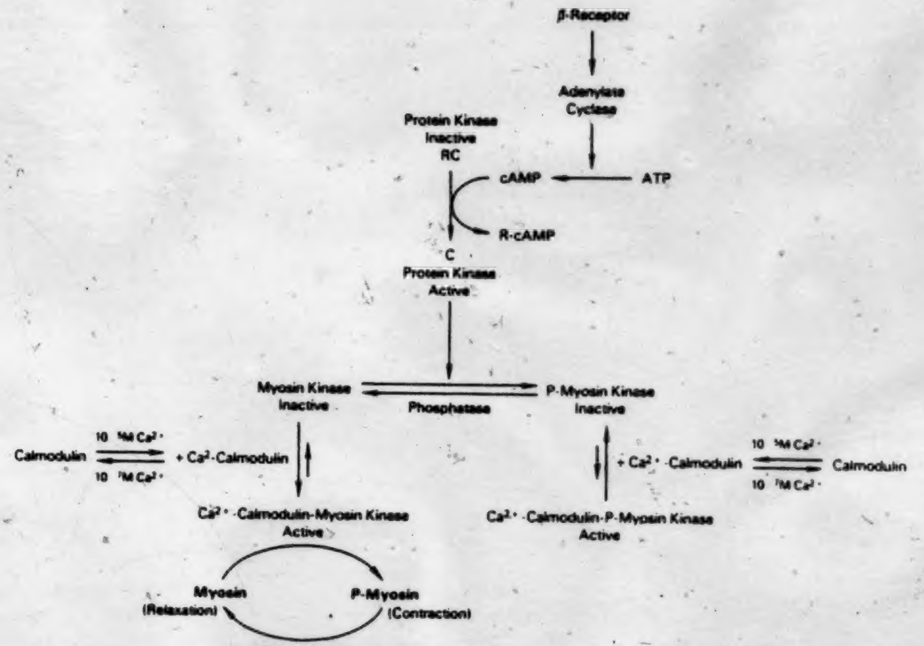

β-Receptor
Adenylate Cyclase
Protein Kinase Inactive RC
cAMP
ATP
R-cAMP
C
Protein Kinase Active
Myosin Kinase Inactive
Phosphatase
P-Myosin Kinase Inactive
Calmodulin
10 ^{5}M Ca^{2+}
10 ^{7}M Ca^{2+}
+ Ca^{2}-Calmodulin
Ca^{2+} -Calmodulin-Myosin Kinase Active
+ Ca^{2+} -Calmodulin
10 ^{5}M Ca^{2+}
10 ^{7}M Ca^{2+}
Calmodulin
Ca^{2+} -Calmodulin-P-Myosin Kinase Active
Myosin (Relaxation)
P-Myosin (Contraction)
Phosphatase

Figure 7. Schematic diagram of the phosphorylation theory of regulation of actomyosin-ATPase in smooth muscle and its modulation by cyclic-AMP [Reproduced with permission from the Annual review of Biochemistry 1980; 49:928, Adelstein RS (Annual Reviews Inc.)]

4. Relaxation follows a return of the sarcoplasmic Ca^{2+} to below the activation threshold with resultant inactivation of the myosin light chain kinase.
5. Phosphorylated myosin is dephosphorylated by one or more phosphatases satisfying the requirements for the reversibility of the system.

The myosin light chain kinase is composed of two sub-units(64). The larger sub-unit has a relative molecular mass ranging from 80,000 to 125,000 depending on the source of the enzyme. The smaller, regulatory sub-unit has been identified as calmodulin. The Ca^{2+} dependency of myosin light chain kinase is determined by the calmodulin. Calmodulin is a single chain of 148 amino acids with a relative molecular mass of 16,700, and has four Ca^{2+} binding sites(65). The molecule appears to be folded into four roughly matching domains, each of which has a Ca^{2+} binding site. On binding Ca^{2+} the calmodulin molecule takes on a new more compact shape and so becomes activated. This in turn activates the larger sub-unit of myosin light chain kinase which can be regarded as the apoenzyme. Both skeletal and cardiac muscle contains a myosin light chain kinase, the larger sub-unit being different from that of the smooth muscle enzyme. Although myosin is phosphorylated by this enzyme in striated muscle, the role of this phosphorylation is not yet clear.

It has been observed that myosin light chain kinase, can itself be phosphorylated by a cyclic-AMP dependent protein kinase. Two sites of phosphorylation on the larger sub-unit of the enzyme have been identified, and termed site A and site B. In the absence of bound calmodulin, both sites are phosphorylated by the protein kinase, whereas only site B is phosphorylated when calmodulin is bound to the apoenzyme. The phosphorylation of Site A leads to diminished binding affinity for calmodulin, which is reflected by a diminished myosin light chain kinase activity(63). Smooth muscle relaxant activity of cyclic-AMP is in part due to the above events. Cyclic-AMP is of course, also believed to act by lowering the free ionic Ca^{2+} concentration in the sarcoplasm.

Dephosphorylation of phosphorylated myosin light chains is performed by a number of enzymes named myosin light chain phosphatases (MLCP). The activity of these enzymes are not affected by the concentration of Ca^{2+}, thus they are active during both the relaxed and contracted states in smooth muscle. During contraction of smooth muscle nett phosphorylation of the light chains occur as the myosin light chain kinase activity swamps out the activity of the phosphatases. When the sarcoplasmic Ca^{2+} concentration falls during relaxation, the light chain kinase activity is inhibited, shifting the nett activity back in favour of dephosphorylation. The different phosphatases found in smooth muscle differ in their sub-unit number and size, with the total relative molecular mass varying from 40,000-160,000.

Biochemical, physiological and pharmacological evidence in favour of the phosphorylation theory has been recently reviewed by Walsh(66). It has been established in a variety of smooth muscle that actomyosin-ATPase activity and the initiation of the contractile process are associated with phosphorylation of the light chains. However, these findings are not adequate to conclude that phosphorylation is the only event accompanying activation of the contractile apparatus. Further evidence in favour of the phosphorylation theory was recently reported by Hartshorne, Walsh and co-workers(57). These workers subjected calmodulin dependent myosin light chain kinase from turkey gizzards to a limited digestion with α-chymotrypsin. This generated a kinase fragment (relative molecular mass:80,000) that was fully active in the absence of free Ca^{2+} ions. Phosphorylation of the myosin in the absence of Ca^{2+} allowed dissociation of phosphorylation from other potential Ca^{2+} dependent regulatory mechanisms. Ca^{2+} independent myosin phosphorylation resulted in a loss of Ca^{2+} sensitivity of the actin activated actomyosin-ATPase activity in crude actomyosin preparations, and of the tension development in skinned smooth muscle fibres.

Although there is considerable evidence in support of the phosphorylation theory, some experimental findings remain unexplained(63).

1. The level of myosin phosphorylation does not always parallel the degree of actomyosin-ATPase activity.
2. In some smooth muscle preparations, although phosphorylation precedes the tension development, the degree of phosphorylation declines considerably prior to steady state force and the maximum number of attached cross-bridges are attained. Furthermore, the maximum active force can be maintained for a long period of time in spite of the degree of phosphorylation declining to a level only slightly above that in the resting tissue.

These results have been explained by the proponents of the phosphorylation theory as indicating that the degree of phosphorylation reflects the velocity of shortening (and thus the cross-bridge cycling rate) in the muscle rather than the degree of tension attained. In addition, the formation of non-cycling cross-bridges or latch bridges have been proposed. This type of cross-bridge could account for the low cost of energy maintenance in smooth muscle. However, no conclusive evidence for the above is available at present.

Leiotonin Theory

This theory was put forward by Ebashi and co-workers in 1975 with the discovery of a 80,000 relative molecular mass protein component of native actomyosin from the chicken gizzard(67). This protein, named leiotonin, was essential for activation of actomyosin in the presence of Ca^{2+} ions. It differed from troponin of striated muscle in that its affinity for actin was greater than that for tropomyosin. Nevertheless, tropomyosin was also required for the full activation of actomyosin-ATPase activity. Leiotonin also differed from troponin in that pure actin and pure myosin from chicken gizzard did not have any ATPase activity, even in the presence of Ca^{2+} ions unless the regulatory proteins leiotonin and tropomyosin were also present. As the leiotonin is located in the thin filaments this theory differs from the phosphorylation theory which is a myosin (thick) filament-linked regulatory system. Myosin light chain phosphorylation is not involved in this leiotonin theory as summarised by Ebashi(68). The essential features of this theory are (Fig. 8):

1. Leiotonin, and tropomyosin specific for smooth muscle (skeletal tropomyosin cannot be a substitute), constitute the regulatory system.
2. Leiotonin is made up of two sub-units: leiotonin A, the regulatory moiety (relative molecular mass: 80,000) and leiotonin C, the Ca^{2+} binding moiety (relative molecular mass: 17,000).

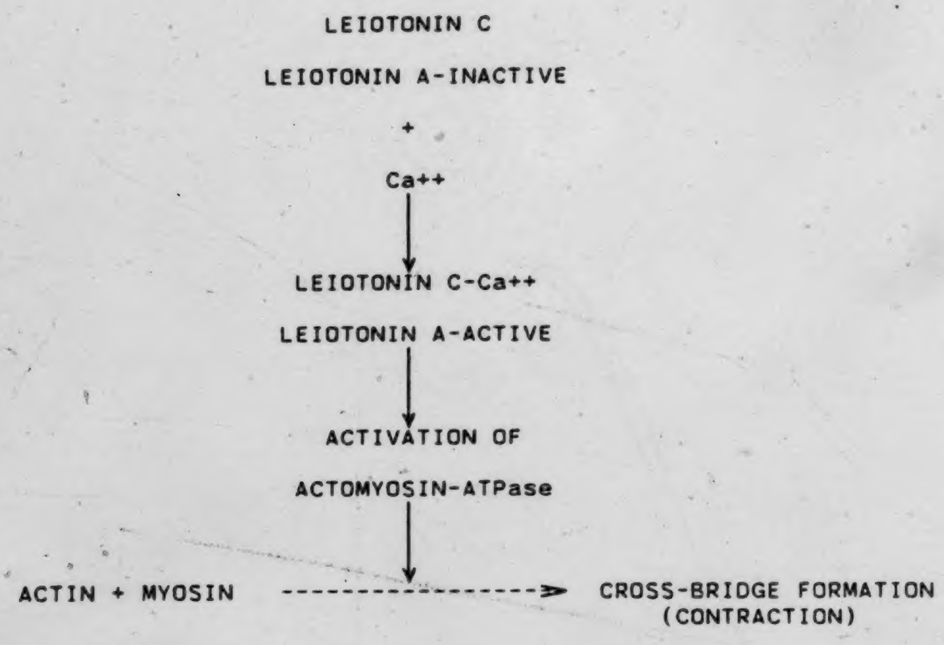

LEIOTONIN C
LEIOTONIN A-INACTIVE
+
Ca++
LEIOTONIN C-Ca++
LEIOTONIN A-ACTIVE
ACTIVATION OF
ACTOMYOSIN-ATPase
ACTIN + MYOSIN
CROSS-BRIDGE FORMATION
(CONTRACTION)

Figure 8. Summary of the leiotonin theory of regulation of actomyosin-ATPase in smooth muscle.

3. Leiotonin C is a Ca^{2+} binding protein, which, although clearly different from calmodulin, can be substituted for by calmodulin in the activation of the actomyosin-ATPase by leiotonin A. However, leiotonin C cannot substitute for calmodulin in activating enzymes.
4. The effective leiotonin/actin molar ratio is less than 1:50.
5. Leiotonin has no affinity for tropomyosin, but only for actin.
6. During contraction or superprecipitation (an in vitro analogue of contraction) of smooth muscle, Ca^{2+} binds to leiotonin C. This allows the activation by leiotonin A of the actomyosin-ATPase activity resulting in the actin-myosin interaction.

In addition to providing evidence for the leiotonin theory Ebashi and co-workers have presented the following evidence which argues against the phosphorylation theory(69). (1) Leiotonin, while fully activating the actomyosin-ATPase activity of a mixture of actin, myosin and tropomyosin in the presence of Ca^{2+}, phosphorylates myosin only weakly. On the other hand, a mixture of myosin light chain kinase and calmodulin phosphorylates the myosin completely, but only weakly activates the ATPase. Thus, the phosphorylation of myosin appear to be dissociated from the actomyosin-ATPase activity in these experiments. (2) When a phosphatase was added to native actomyosin superprecipitation occurred in the presence of Ca^{2+} ions without any phosphorylation of the myosin light chains. (3) The pH dependency of this system also provided evidence against the phosphorylation theory. At pH 6.7 the ATPase was fully active with only minimal phosphorylation.

The mechanism of action of the leiotonin is not clear at present. As leiotonin is functional at a very low leiotonin: actin ratio, it is believed that its role is unlikely to be structural. If this is proven it would be another difference from the striated muscle regulatory protein, troponin, which plays a structural role in the activation actomyosin-ATPase. This low (1:50) leiotonin: actin ratio has been used as an argument against the leiotonin theory as the effective troponin:actin ratio is 1:7. However, two other proteins, β-actin and gelsolin have been found to be effective at a molar ratio (to actin) of less than 1:50(68). These two proteins do not require tropomyosin for effective function whereas tropomyosin is required for activation of ATPase by leiotonin. The exact role of tropomyosin in the leiotonin theory is not well understood.

In summary, there seems to be good evidence for and against both phosphorylation and leiotonin theories. It is possible that both regulatory mechanisms may be functional in smooth muscle either in different muscles or in a single muscle as a dual regulatory system.

AUTONOMIC INNERVATION OF VASCULAR SMOOTH MUSCLE

Autonomic nerves have been identified in the walls of most blood vessels(70). Most of the nerve fibres are sympathetic noradrenergic in origin although sympathetic cholinergic fibres are found in some vessels such as the skeletal muscle vascular bed. A parasympathetic supply is present in other blood vessels such as the vasculature of the erectile tissues and in cerebral circulation(71). More recently, the existence of purinergic nerves in non-vascular smooth muscle has been documented (72). On the other hand, some blood vessels such as the umbilical artery and the human deep limb veins are not innervated. The latter contains smooth muscle and contracts in response to noradrenaline: therefore the demonstration of adrenergic receptors cannot be taken as indicating the presence of an adrenergic nerve supply(70). As a great deal more is known about the adrenergic nerve supply compared to the other two types the ensuing discussion relates to the former unless specified otherwise.

The adrenergic nerves to blood vessels are non-myelinated, post-ganglionic fibres. These fibres usually form two plexuses in the adventitia: a primary plexus in the middle or outer third of the adventitia and a terminal plexus typically restricted to the adventitio-medial junction(73). This adrenergic terminal ground plexus tends to be similar to that found in non-vascular smooth muscle, being irregular, multiaxonal and of varying density. Non-myelinated axons, 0.25-0.5 µm in diameter with a surrounding schwann cell sheath form the plexus, which surrounds the tunica media like a sheath. The axons contain varicosites 1.5-2.0 µm in diameter at intervals of 3-10 µm along their lengths. These are the storage and release sites for noradrenaline which is contained in a large number of storage vesicles within the varicosites. A nerve impulse propagating along an axon successively depolarizes a series of variocosities resulting in the release of transmitter at each of these sites. The released neurotransmitter brings about changes in vascular smooth muscle tone. The nerves themselves terminate at the adventitio-medial junction or in the outer third of the media in most vessels. A model for the innervation of smooth muscle has been proposed by Burnstock on the basis of electrophysiological, histochemical and electron-microscopical studies(74). The essential features of this model are as follows (Fig. 9):

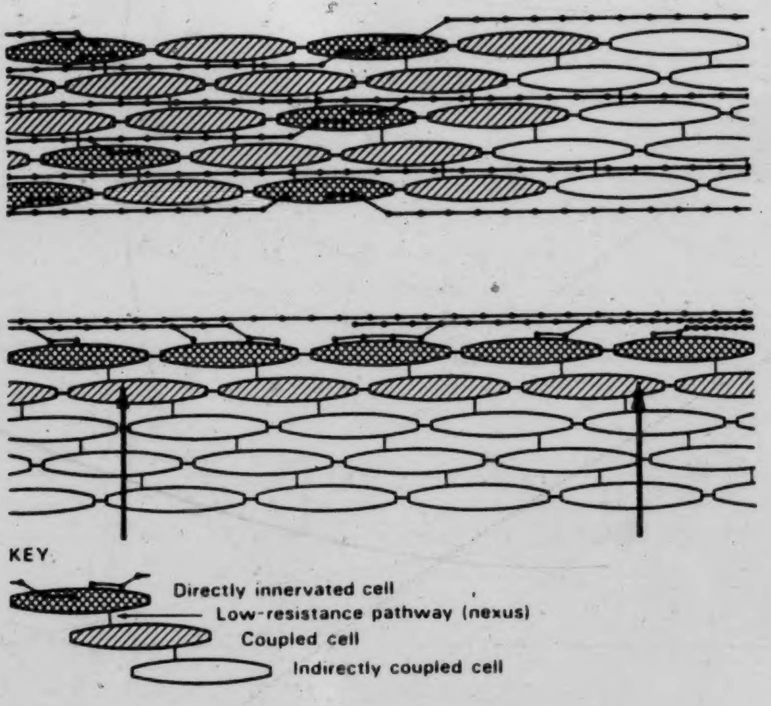

KEY
Directly innervated cell
Low-resistance pathway (nexus)
Coupled cell
Indirectly coupled cell

Figure 9. Model of the vascular smooth muscle neuromuscular junctions. Vascular smooth muscle is controlled by both nerves (—) and circulating catecholamines (arrows) and contains three types of cells (1) directly innervated cells (2) directly coupled cells (3) indirectly coupled cells [Reproduced with permisssion from Burnstock G, British Medical Bulletin, (74)].

1. The effector unit is a muscle bundle rather than a single smooth muscle cell.
2. Unlike those of the skeletal muscle neuromuscular system, autonomic nerves in smooth muscle run long distances, containing varicosities which have high levels of neurotransmitter: the transmitter is released en passage during the conduction of an impulse.
3. Individual muscle cells are connected by low-resistance pathways or gap junctions which allow electrotonic spread of activity within the effector bundle.
4. In most smooth muscle bundles, some (but not all) muscle cells are directly innervated, i.e. in close (20-120 nm) apposition with the nerve varicosites, and are directly affected by the transmitter released from them ("directly innervated cells"). The adjoining cells have been named "coupled cells" as they are electrotonically coupled to the former by low resistance pathways so that excitatory junction potentials can be recorded in these cells. These junction potentials are very slow (nearly 1.0s), resulting in a whole area of the effector bundle depolarizing almost simultaneously, triggering an action potential. The action potential propagates through the effector bundle to activate a third group of cells named the "indirectly coupled, cells" which are neither directly innervated nor directly coupled and yet respond on stimulation of the nerves supplying the organ.

The density of innervation tends to vary widely in different parts of the vascular system. In general, arteries receive a richer innervation than veins with large arteries having a relatively poor nerve supply compared to small arteries and arterioles. Further, there is considerable species variation in the innervation of the same type of blood vessel. Rat aorta is sparsely innervated, whereas the rabbit aorta contains a terminal plexus at the adventitio-medial junction. The large elastic arteries usually do not contain nerves penetrating the tunica media. Muscular arteries such as the rabbit ear artery tend to be more heavily innervated than elastic arteries. However, the innervation is usually confined to the adventitio-medial junction in most muscular arteries. In some muscular arteries, particularly in certain species such as sheep and man, the nerve fibres penetrate about one-third of the thickness of the media. Although veins in general have an innervation which is less dense than arteries, some medium sized veins such as the small saphenous vein of the rabbit have an extensive medial innervation. Medial innervation is commonly found in subcutaneous limb veins. A summary of the innervation characteristics in a variety of blood vessels is provided by Bevan et al.(73) who also make the following points regarding neuronal density in vascular smooth muscle.

1. Nerve density does not vary systematically with vessel diameter or wall thickness.
2. The same vessel may show a widely different pattern of innervation depending on the exact location at which the morphology is investigated, e.g., the first few millimetres of the rabbit saphenous artery is not innervated. It then gives off a small muscular branch after which the innervation becomes dense and medial in nature. Passing distally, the innervation takes on an adventitio-medial pattern. Thus, when blood vessels are

excised for in vitro studies, they have to be removed from the same anatomical site.

3. Nerve density, provided other parameters of transmission are similar, can be related to the maximum level of neurogenic tone of which a vessel is capable. However, there are exceptions to this rule.
4. Nerve density does not remain constant with age.
5. Neuronal density of a particular vascular segment is not the same in different species.

Autonomic innervation of the canine lateral saphenous vein

An investigation on the innervation of this vein using fluorescence microscopy (with formaldehyde) and autoradiography (with tritiated noradrenaline) was reported by Osswald and co-workers(17,18,75). This study demonstrated the presence of adrenergic nerve fibres spread across the whole media. The nerve fibres were unmyelinated axons enwrapped by Schwann cells. Sometimes the axoplasm was seen to contain microtubules only, but in most cases it contained numerous dense-core vesicles (made up of large granular and small granular vesicles) agranular vesicles and mitochondria. The large granular vesicles constituted approximately 43 per cent of all dense-core vesicles and were 80-200 nm in diameter whereas the small granular vesicles were 40-60 nm in diameter. This considerable proportion of large granular vesicles (43 per cent) found in this vein is different from most adrenergic nerves, which contain predominantly small dense core vesicles. The diameter of the large dense-core vesicles observed is also larger (80-200 nm) than in most adrenergic nerves. The agranular vesicles present had a diameter ranging from 70-200 nm. The areas of the axons containing dense-core vesicles were often found to be partially lacking Schwann cell sheaths and were located in close apposition to smooth muscle cells. The neuromuscular distance at these points usually varied from 100-300 nm with a much shorter distance (20 nm) sometimes being observed rarely. There was no thickening of the opposing plasma membranes at these neuromuscular junctions, however, the basement membranes were continuous at these points. This narrow cleft width observed in the saphenous vein accounts for the predominant role played by uptake_1 in the transmitter disposition in this vein. It may also account for the relatively short delay (less than a second) between the commencement of electrical stimulation and the beginning of the contractile response, observed in this preparation.

The sympathetic innervation to the veins of the dog hind-limb originates from the lumbar spinal cord(76,77). The nerves leave the cord via the upper lumbar roots and run in the main sympathetic ganglia. The nerves join the sciatic nerve via the rami to the sixth and seventh lumbar nerves and the second sacral nerves. Thus, surgical sympathetic denervation of the veins in the hind-limb is achieved by dividing the sympathetic chain at the second or third lumbar vertebral body and dissecting the chain free from the surrounding tissues to the level of the fifth lumbar vertebral body(78).

ELECTROPHYSIOLOGY OF SMOOTH MUSCLE

Vascular smooth muscle cells, like all other living cells, maintain a potential difference across their cell membranes, with the inside being negative compared to the outside. This resting membrane potential (Em) varies from -40 to -75 mv in different smooth muscle cells. This potential difference is dependent on two factors. (1) The unequal distribution of ions across the sarcolemmal (cell) membrane with different permeabilities to the respective ions. (2) The electrogenic transport of ions across the cell membrane with the aid of the sodium/potassium membrane pump (Na^+/K^+ pump).

Resting membrane potential

The unequal distribution of ions across the sarcolemma results in the generation of a diffusion potential for each ion depending on its concentration difference across the sarcolemma and the membrane permeability to that particular ion. If one assumes an unrestricted movement of a particular ion, the diffusion potential (at which there is no nett movement of the ion across the sarcolemma) is given by the Nernst equation.

$$E_I = \frac{RT}{zF} \ell n \frac{[I]o}{[I]i}$$

E_I : equilibrium diffusion potential for ion I

R : gas constant (8.316 joules per degree)

T : absolute temperature

z : valency of the ion

F : Faraday constant (96,500 coulombs/mol of ion)

ℓ_n : natural logarithm (2.313)

[I]o : concentration of ion in extracellular fluid

[I]i : concentration of ion in intracellular fluid

At a temperature of 30°C the Nernst equation for the K^+ ion can be simplified to:

$$E_m = \frac{8.316 \times 303}{96500} \quad 2.3 \log_{10} \frac{[K+]o}{[K+]i} \quad \text{Volts}$$

$$= 60 \log_{10} \frac{[K+]o}{[K+]i} \quad \text{mV}$$

Thus the membrane potential would depend on the resultant effect of all the diffusion potentials for the different ions in the muscle cell and the extracellular fluid. However, the potential difference each ion contributes to the Em depends on the permeability of the sarcolemma to that particular ion, in the resting state. This is not taken into account in the Nernst equation which assumes unrestricted movement of the ions. The resting membrane potential in the presence of a group of ions (e.g. Na^+, K^+, Cl^-) across the cell membrane is given by the constant-field equation of Goldman(79).

$$E_m = 60 \log_{10} \frac{P_K (K^+)o + P_{Na} (Na^+)o + P_{Cl} (Cl^-)i}{P_K (K^+)_i + P_{Na} (Na^+)_i + P_{Cl} (Cl^-)_o}$$

P_K, P_{Na}, P_{Cl}:membrane permeabilities for K^+, Na^+ and Cl^-

If a membrane were selectively permeable to K^+ the membrane potential would be approximately equal to the equilibrium diffusion potential for K^+ across the membrane (the Goldman equation can be equated to the Nernst equation at this point). Such a membrane would show a 60 mV change per 10 fold change in $[K^+]o$ that would be expected theoretically from the equations given above(80). However, the average slope of the Em versus log $[K^+]o$ curve (measured between 10 and 100 mmol $[K^+]o$) ranges between 32 and 48 mV/decade. This discrepancy is believed to be due to two factors(80,81). First, the assumption that the membrane is selectively permeable to K^+ is in fact not true as the cell membrane is permeable to both Cl^- and Na^+, although to a much smaller extent. The permeabilities to these two ions are higher in smooth muscle compared with skeletal muscle. Secondly, as $[K^+]o$ is increased in vascular smooth muscle, K^+ conductance (g_K) increases (g_K: the conductance of an ion through a membrane is the reciprocal of its resistance — It is defined as the nett current flow per unit voltage)(80). Such an increase in g_K tends to hyperpolarize the membrane at any given elevation in $[K^+]o$. In other words, the amount of depolarization produced by a given elevation is $[K^+]o$ would be less because of the increase in g_K. This factor tends to be more prominent in arterial smooth muscle cells which have a low g_K and P_K since E_m (the membrane potential) is further from E_K (the K^+ equilibrium potential).

Na^+/K^+ membrane pump

As explained above, the resultant equilibrium diffusion potential for a group of ions can be calculated using the Goldman equation. The resultant value obtained in smooth muscle often tends to be less negative than the actual membrane potential (E_m) recorded by electrophysiological studies. The difference between the two values is believed to be contributed by the electrogenic Na^+/K^+ pump. A major problem with the approach is that the data for ion concentrations and transmembrane ion permeabilities that are required for calculation of E_m in the Goldman equation are difficult to obtain accurately. The Na^+/K^+ pump of smooth muscle allows the cells to extrude Na^+ in exchange for extracellular K^+. The inward diffusion of Na^+ down its electrochemical gradient and the outward diffusion of K^+ along its concentration gradient necessitates the existence of the Na^+/K^+ pump to maintain the intracellular ion composition relatively constant(82). If the exchange of the two ions were one for one, the pump would not contribute to any nett shift of current across the membrane, i.e., the pump would be electrically neutral. However, in most cases the exchange is not one for one, more Na^+ ions being extruded than K^+ ions brought in, i.e., the pump is electrogenic. The coupling ratio for the pump in red blood cells is $3Na^+:2K^+$. Estimates in other tissues* though variable, generally approximate the 3:2 ratio. The pump is energy dependent and is associated with the membrane bound enzyme Na^+/K^+-ATPase which hydrolyses the Mg-ATP. The presence of Mg-ATP inside the cell is essential for the activity of the pump(79) with one molecule being hydrolysed for every three Na^+ ions pumped out. The level of cell ATP can be lowered by removal of substrate from the bathing medium (O_2 and glucose being replaced by N_2 and sorbitol). Since smooth muscle cells are capable of anaerobic respiration a combination of iodacetate and dinitrophenol is required to reduce cell ATP to unmeasurable levels. Under these circumstances cells rapidly lose K^+ and gain Na^+(83). The activity of the Na^+/K^+ pump is a function of the intracellular Na^+ concentration and the extracellular K^+ concentration, the pump being inhibited if the K^+ concentration of the bathing medium is made zero. It is also inhibited at low temperatures because of its energy dependency. Cardiac glycosides (e.g., ouabain, digitalis) inhibit the pump if added to the external medium by inhibition of the enzyme Na^+/K^+-ATPase.

There is considerable debate as to the magnitude of the contribution of the Na^+/K^+ pump to the resting membrane potential in smooth muscle. Two approaches have been used to estimate this contribution(82). The first method involves the determination of the difference between the recorded membrane potential and that calculated using the Goldman equation as explained above. The second method involves the determination of the change in membrane potential during the inhibition of the pump. Both methods are not without error(82) making the quantification of the contribution of the pump to the resting membrane potential difficult. The contribution is probably much less than was formerly believed amounting to about 4mV(84).

In smooth muscle, where tension is a function of the membrane potential, the Na$^+$/K$^+$ pump may function as a mediator of the vasoconstriction and vasodilation associated with certain "drugs"(82). Hyperkalaemia and hypokalaemia (moderate) are associated with vasodilatation and vasoconstriction respectively. This is probably mediated through modulation of the activity of the Na$^+$/K$^+$ pump in the muscle cells. Thus, high extracellular K$^+$ leads to stimulation of the pump with resultant hyperpolarization and a decrease in tension. These responses are antagonised by ouabain. Shepherd and co-workers showed that the Na$^+$/K$^+$-ATPase inhibitor acetylstrophanthidin potentiated the contractions of the lateral saphenous veins produced by noradrenaline, 5-hydroxytryptamine, acetylcholine and barium(85). These effects of cardiac glycosides are probably due to changes in resting membrane potential(82).

The inwardly directed Na$^+$ gradient created by the Na$^+$/K$^+$ pump is used in transporting other ions or molecules against their concentration gradients. The Na$^+$/Ca^{2+} exchange, Na$^+$/amino acid exchange and Na$^+$/Mg^{2+} exchange are some of the transport processes in smooth muscle, which are believed to function using this Na$^+$ gradient(83). The Na$^+$/K$^+$ pump may also play a role in the post-junctional supersensitivity in smooth muscle. Thus a decrease in Na$^+$/K$^+$-ATPase activity with a partial depolarization of the smooth muscle cell membrane leads to the hyperreactivity in some cases of post-junctional supersensitivity(82).

Action potentials

Action potentials of different configurations have been recorded in a wide variety of blood vessels(81) and they occur in bursts or at regular intervals. The action potentials may resemble those in skeletal muscle with a sharp rise and fall or they may have a prolonged time course with a plateau phase similar to that observed in cardiac muscle, e.g. turtle aorta. The spike potentials in smooth muscle may have an overshoot of up to 20 mV. However, the maximum rate of rise of the spike is very much slower (5-20 V/s) compared to striated muscle or nerves (1000 V/s). The maximum rate of repolarization is often similar to that of depolarization or it may be slightly faster(84).

Some smooth muscles such as in the guinea-pig urinary bladder and in the porto-mesenteric veins of many species, exhibit spontaneous action potentials. Others demonstrate action potentials only in response to stimulation with drugs. Tetraethylammonium ion (TEA) which reduces K^+ conductance (gk) induces spontaneous action potentials in some smooth muscle with no inherent activity. In other smooth muscles which do not respond to transmural nerve stimulation with action potentials, it induces spikes in response to stimulation(80).

The ionic basis of the action potentials in smooth muscle has been the subject of some debate(81). As vascular smooth muscle action potentials have a variety of configurations with different time courses, it is possible that the ionic mechanisms may be different in different muscles. In the majority of preparations reduction of the extracellular Na^+ concentration does not have an appreciable effect on the action potentials in smooth muscle. This same manouevre causes a reduction in the rate of depolarization and the amplitude of the overshoot in nerves and skeletal muscle. This argues against Na^+ ions playing a major role in the action potentials in smooth muscle. The lack of effect of the fast Na^+ channel inhibitor tetrodotoxin on the action potential supports the above concept. The smooth muscle spikes are blocked by the bivalent ions cobalt (Co^{2+}), nickel (Ni^{2+}) and Manganese (Mn^{2+}). They are also abolished by the removal of Ca^{2+} from the extracellular fluid and by calcium anatagonists. Both strontium (Sr^{2+}) and barium (Ba^{2+}) ions are able to replace Ca^{2+} as carriers of the inward current(84). The above findings point to Ca^{2+} ions being the major ion involved in action potentials of vascular smooth muscle. However, in the quiescent sheep carotid artery, Keatinge was able to induce electrical activity in Ca^{2+} and Mg^{2+} free solutions(86). These action potentials were abolished when extracellular Na^+ was replaced by Tris or choline, and they also ceased when Ca^{2+} or Mg^{2+} were added. It was concluded that Na^+ was the principal ion carrying the depolarization current in the action potential in this preparation. However, tetrodotoxin, the fast Na^+ channel blocker, did not abolish these action potentials.

In addition to action potentials, some smooth muscle demonstrate a regularly occurring, rather slow changes in membranes potential (lasting up to several seconds) which are referred to as slow waves(84). These slow waves vary in frequency and time course from one tissue to another and may not necessarily exceed the threshold to trigger action potentials. Slow waves are present in intestinal smooth muscle of many species and their amplitude is generally about 20 mV, though it may be up to 40 mV rarely. They may occur without any accompanying changes in tension. The slow waves increase muscle tension either if the waves crosses the threshold and initiates (one or more) action potentials or if they cross the contraction threshold in gradedly responsive muscle (see below). Unlike action potentials, slow wave activity is little affected by changes in the membrane potential and they are also not propagated in the tissue(87). The depolarization phase of the slow waves are believed to be mediated via a Na^+ influx which is resistant to tetrodotoxin. There is good evidence that these slow waves are a function of spontaneous changes in electrogenic pumping(82). Ouabain, K^+-free solutions and cooling inhibits slow waves, supporting the role of the Na^+/K^+ pump in the generation of these waves.

Electrophysiologically smooth muscle can be divided into two broad types

(1) spike generating smooth muscle

(2) non-spike generating smooth muscle.

The spike-generating smooth muscle can be sub-divided into the single-unit type and the multi-unit type on the basis of their ability to generate and propagate electrical activity(81). In both single-unit and multi-unit spike generating smooth muscle, the contractions are brought about by action potentials. However, in the single-unit variety, the action potentials are triggered by slow-wave depolarizations or pacemaker potentials resembling the phase 4 depolarization of cardiac muscle, but having a much slower time course. The portal-mesenteric veins of many species provide a good example of this type of vascular smooth muscle. Smooth muscles of the intestine and urinary tract are other examples of single-unit spike generating smooth muscle. The action potentials may occur in bursts or at regular intervals in this type of smooth muscle and they travel along the tissue at a conduction velocity of 10-80 mm/s via low resitance pathways between the muscle cells. However, the spread is usually decremental. Therefore large preparations show multiple pacemaker sites and variable patterns of contraction(81). These action potentials originate within the muscle itself as they are unaffected by tetrodotoxin. The contractions in this type of muscle is triggered by the action potentials, with the basal tone in the muscle being proportional to the frequency of the spikes in the resting state. The frequency of the action potentials tends to be decreased by cooling. On the other hand, the frequency is increased in some tissues by stretching of the muscle. This may provide a possible basis for myogenic autoregulation of blood flow. Neurotransmitters released at nerve endings within the muscle and exogenous drugs can also modulate the frequency of the spikes with either an increase or decrease depending on the agent involved. These drugs may exert their action by changing the membrane potential: depolarization causes an increase and hyperpolarization a decrease in frequency or the abolition of spontaneous activity(84). This is probably due to the fact that raising or lowering the membrane potential would increase or decrease (respectively) the time required for the spontaneous depolarization wave to reach threshold potential, thus altering the frequency of the spikes. Stimulation of portal-mesenteric veins by noradrenaline and adrenaline has been shown to result in an increase in the frequency of action potentials(81,88). With high concentrations of these agents, the marked depolarization produced, obliterated the action potentials. However, the strong contractile response remained intact. On the other hand, the action of isoproterenol in portal veins of different species was not as clear cut as the above(81). The drug caused hyperpolarization with cessation of spontaneous activity in the rabbit and guinea pig portal veins. However, in the rat portal vein, isoproterenol resulted in the action potential pattern changing to one of short, frequent bursts, each containing fewer spikes associated with depolarization of the cell membrane. It is possible that isoproternol may have other actions which alter the relaxatory mechanisms in smooth muscle more directly via the β-receptors and cyclic AMP.

In the multi-unit variety of spike generating smooth muscle action potentials are initiated by transmitter induced excitatory junction potentials. The transmitter released at nerve endings produce miniature excitatory junction potentials at the post-synaptic membrane. These miniature junction potentials summate to produce an all-or-none action potential when the membrane potential reaches threshold. Mouse vas deferens typifies this type of smooth muscle. There are no good examples of vascular smooth muscles with similar electrical properties and predominance of neural control(81).

Non-spike generating vascular smooth muscle contract in the absence of action potentials. This muscle is characterised by graded depolarizations and contractures instead of spikes and twitches. The contraction is accompanied by a membrane depolarization without a true action potential with the contraction being maintained as long as the membrane remains depolarized. A true cause and effect relationship between the electrical and mechanical events is more difficult to prove in this type of smooth muscle: it is possible that the two responses just occur as simultaneous parallel phenomena. Rabbit pulmonary artery provides an example of this type of vascular smooth muscle. In smooth muscle, which shows a graded response, electrotonic spread of the depolarization produced by neurotransmitters probably does not play a significant role in the cell-to-cell spread of activity. The absence of action potentials is probably due to the fact that the mechanisms necessary for the fast, regenerative changes in ion conductance that mediate spikes are, in fact, not normally operating in the membranes of these cells(81). However, some smooth muscle of this type can be 'stimulated' to produce action potentials by the tetraethylammonium ion.

In both spike-generating and non spike-generating muscles, an inward Ca^{2+} current occurs during the depolarization phase via Ca^{2+} channels. There appears to be a number of voltage sensitive Ca^{2+} channels in smooth muscle: rapidly activating and inactivating channels involved in the upstroke of the action potentials, and slowly activating but maintained Ca^{2+} channels functional during prolonged depolarization. Drugs which produce depolarization by a mechanism other than an inward Ca^{2+} current may also secondarily activate any voltage-sensitive Ca^{2+} channels if the membrane potential is moved into the correct range. This would generate tension in the smooth muscle by increasing the Ca^{2+} influx. The exact mechanisms by which drugs influence the membrane potential have not been established in most cases. In principle they probably act primarily through an effect on passive ion permeabilities and in some instances electrogenic ion transport(80).

Electro-mechanical and Pharmaco-mechanical Coupling

In both spike generating and non spike-generating muscle described above, the contraction of the muscle cells is coupled with an electrical event at the cell membrane: an action potential in the former and a graded depolarization in the latter. This type of coupling is referred to as electro-mechanical coupling and is similar to the phenomenon found in skeletal and cardiac muscle. A quantitative relationship between the membrane potential and the tension can be demonstrated in skeletal and cardiac muscle after blockade of the action potential. An S-shaped relationship has been found defining the mechanical threshold and the characteristics of the electromechanical coupling(81). These curves are shifted by drugs and ions. Attempts at similar characterisation in smooth muscle has been less successful because of the difficulty in blocking the action potential without losing the contractility(89). However, experiments carried out at 10°C were more successful in demonstrating a S-shaped relationship between tension and the extracellular K^+ concentration in the rat portal vein(90). The fact that normally spike generating vascular smooth muscle maintain tonic contractures in depolarizing high K^+ solutions demonstrates that the action potentials are not essential for evoking contraction in these tissues.

Although changes in membrane potential can initiate changes in tension, in smooth muscle certain hormones and drugs can cause contraction without any change in the membrane potential. This phenomenon known as pharmaco-mechanical coupling was first described by Somlyo and Somlyo in 1968(91). Pharmaco-mechanical coupling has been defined as a process or processes through which drugs can cause contraction or relaxation of smooth muscle without a necessary change in the resting membrane potential or in action potential frequency. This definition does not imply that pharmacomechanical coupling cannot also act concomitantly with a change in the membrane potential. It is quite probable that electro-mechanical and pharmaco-mechanical coupling processes contribute to activation (or inhibition) simultaneously. Quantitative investigations are needed to ascertain the relative magnitudes of the two processes. For instance, if the contraction produced by an agonist which also produces a simultaneous depolarization is greater than that elicited by an equivalent depolarization by high K^+ solution (or an electrical current), it is probable that the excitatory actions of the agonist is due to both electromechanical and pharmaco-mechanical coupling (or to pharmaco-mechanical coupling alone). Farley and Miles in recent experiments carried out in the dog trachealis muscle, demonstrated that although acetylcholine in a concentration of 10^{-7} mol/l produced some membrane depolarization with a contraction approximately 15 per cent of the maximum, an equivalent depolarization by high K^+ did not generate tension(92). Further, a recent study on the guinea-pig main coronary artery demonstrated that the contractions caused by acetylcholine were accompanied by a membrane hyperpolarization(93). The evidence for pharmaco-mechanical coupling has been summarised as follows by Johansson and Somlyo(81):

1. the same blocking and potentiating agents are effective in polarized as well as depolarized smooth muscle.
2. depolarization by drugs may be less, but the maximum contraction greater than the respective effects of high K^+ solutions.
3. the differences in the maximum contractile effects of different drugs are maintained after depolarization.
4. drug-induced contractions may be sustained in smooth muscles that respond to depolarization by high K^+ with a transient phasic contraction.

5. relaxing agents can relax polarized smooth muscle without evidence of hyperpolarization or inhibition of spike electrogenesis.

These authors comment that 'whereas theoretically the possibility of excitatory pharmaco-mechanical coupling without depolarization in polarized smooth muscle is possible, a rigorous demonstration of this phenomenon requires experiments in which membrane potential and contractile responses of single cells are monitored simultaneously'. The mechanism by which pharmacomechanical coupling occurs is not clear at present but the suggested possibilities are(81):

1. mediation by the influx of extracellular Ca^{2+}. As by definition the membrane potential should not change during pharmaco-mechanical coupling, for the above mechanism to account for this process there has to be a simultaneous movement of another ion to balance the membrane potential change that would be produced by the influx of Ca^{2+}.
2. mediation via release of Ca^{2+} from the sarcoplasmic reticulum in response to a change in permeability of the surface (plasma) membrane at the couplings between the reticulum and the surface membrane.
3. mediation via a direct effect of the drug on intracellular Ca^{2+} storage sites. It is unlikely that large polypeptides such as angiotensin could act via this mechanism but more permeant molecules such as the prostaglandins could exert intracellular effects.

These mechanisms are not mutually exclusive, thus two mechanisms could occur together. In summary, although all available evidence suggests that pharmaco-mechanical coupling probably occurs in smooth muscle, no absolute proof is available at present.

CALCIUM REGULATION IN SMOOTH MUSCLE(29,30,81,83,94)

As tension development in muscle is a function of the concentration of free intracellular Ca^{2+}, the regulation of the latter is of prime importance in all muscle cells. Compared with striated muscle, very little is known about the sub-cellular regulation of Ca^{2+} in smooth muscle. The specific role of the various Ca^{2+} binding sites (sarcoplasmic reticulum, mitochondria, sarcolemma) is not clear at present. Further, the immediate source of activator Ca^{2+} is not known, nor is the mechanism of relaxation known. Smooth muscle cells are activated when the ionised intracellular Ca^{2+} rises above 10^{-7} mol/l with maximal activity between 10^{-5} and 10^{-4} mol/l(83). The total tissue content of calcium in smooth muscles may, however, be 100 fold of the amount required for maximum contractile activity. The total tissue calcium is made up of extracellular free, extracellular bound, intracellular free and intracellular bound fractions. Brading and Widdicombe estimated these calcium fractions in the taenia coli of the guinea pig(95). The total tissue calcium was found to be 2.73 ± 0.13 mmol/kg fresh weight of tissue (Ca^{2+} in the bathing medium during the experiment = 2.5 mmol/l). If the free Ca^{2+} present in the [^{14}C] sucrose space (used to estimate the extracellular space) was substracted, the Ca remaining in the tissue was 1.8 ± 0.13 mmol/kg fresh weight of tissue. The extracellular bound Ca was estimated by treating the tissue with Lanthanum (La^{3+} 5 mmol/l) for 60 min (this may be an overestimate as some cellular Ca is lost during the La^{3+} treatment). If this amount is subtracted the remaining Ca, which can be considered as the total intracellular Ca, was found to be 0.43 ± 0.09 mmol/kg fresh weight of tissue. This is still an order of magnitude greater than that necessary to activate maximally the contractile apparatus in smooth muscle cells. The bound intracellular Ca is located in the organelles such as the mitochondria, the sarcoplasmic reticulum, the nucleus and in the sarcolemma. In the resting muscle, the concentration of free Ca^{2+} in the myoplasm is less than 10^{-7} mol/l although the concentration of free Ca^{2+} in the extracellular fluid is greater than 10^{-3} mol/l. Thus, for vascular smooth muscle to be relaxed, its plasma membrane must support a 10,000 fold concentration gradient of the Ca^{2+} ion. Recent studies in myocardial tissue by Langer have formed the basis for a model whereby the glycocalx (a coating external to the unit membrane) participates in the transmembrane ionic exchange(96). The glycocalyx is composed of two layers: an inner, less dense, 20 nm thick surface coat and an outer, slightly more dense, 30 nm thick external lamina. A high concentration of negatively charged sites are present in the glycocalyx. Sialic acid residues which form the terminal groups in the oligosaccharide portions of the glycoproteins and glycolipids composing the glycocalyx form a majority of these negatively charged sites. Treatment of myocardial tissue with neuraminidase, which selectively removes sialic acid residues, increases the uptake and washout of radioactive Ca^{2+} fivefold. Further, following this enzymatic treatment, La^{3+} ions, which are normally restricted to extracellular space enters the cell leading to displacement of more than 80 per cent of the exchangeable calcium. Langer proposed that the glycocalyx may be necessary for the prevention of uncontrolled calcium entry from the extracellular space.

Activator calcium in smooth muscle

The Ca^{2+} responsible for activation in smooth muscles comes from intracellular and/or extracellular sources. The source of activator

Ca^{2+} seems to differ between smooth muscles from different vessels and between different stimulants used to initiate contraction. Vascular smooth muscle, which shows spontaneous contractions accompanied by action potentials (spike generating smooth muscle, see above), depend on extracellular Ca^{2+} for this spontaneous activity. The action potentials in the porto-mesenteric veins are blocked by the removal of extracellular Ca^{2+} ions(81). Agonists, which cause depolarization of the cell membrane, lead to entry of Ca^{2+} from the extracellular space through Ca^{2+} channels resulting in muscle contraction(97). K^+ depolarization has been used for the investigation of depolarization induced contraction in smooth muscle. Contractile responses produced by high concentrations of K^+ are considerably inhibited in a Ca^{2+}-free medium lending further support to the role of extracellular Ca^{2+} in this contraction. The contraction in the presence of K^+ consists of two components: an initial, large, transitory phasic component, and a second slower increase in tension which subsequently diminishes to a lower plateau level of tension over 90-120 minutes(98). The latter is referred to as the tonic component. These phasic and tonic components of the contractile responses have been identified in many agonist induced contractions in smooth muscle. The phasic component has been found to be dependent on intracellular Ca^{2+} ions and the tonic component on extra-cellular Ca^{2+}(99). However, both components in the case of the contractile response to K^+ appear to be dependent on extracellular Ca^{2+} ions(98). Thus both components of the response were highly sensitive to the Ca^{2+} channel antagonist verapamil and also inhibited by La^{3+} which competes with Ca^{2+} ions primarily at extracellular sites on the membrane. These findings do not imply that extracellular Ca^{2+} is the only source of activator ions for the contractile apparatus in K^+ induced contraction. It is quite possible that the inflow of Ca^{2+} ions from the extracellular medium may trigger a further release of Ca^{2+} from intracellular Ca^{2+} stores (calcium-induced calcium release). Hurwitz et al(98) suggested that the two components of the K^+ induced response in intestinal smooth muscle are probably mediated via two separate Ca^{2+} channels in the membrane. The preferential inhibition of the phasic component by a lower concentration of La^{3+} (both components being inhibited by a higher concentration of La^{3+}) suggested the above conclusion. It should be clear that the terms phasic component and tonic component are purely descriptive terms used to identify components of agonist induced contractions in muscle. The source of Ca^{2+} responsible for the two components may vary depending on agonist as well as the tissue involved.

The concept of a heterogeneous group of Ca^{2+} channels in the vascular smooth muscle cell membrane has developed over the recent years with the availability of pharmacological agents that inhibit Ca^{2+} entry. It is believed that at least three Ca^{2+} entry channels are present in the sarcolemma(100):

1. the potential operated or potential dependent Ca^{2+} channels (POC or PDC): these channels are activated by a depolarization of the cell membrane produced by agonists, e.g., K^+, depolarizing concentrations of noradrenaline. These potential operated channels are preferentially blocked by organic calcium antagonists such a verapamil, nifedipine, and diltiazem.
2. receptor operated or receptor dependent Ca^{2+} channels (ROC or RDC): these are activated by agonists without any accompanying

change in the membrane potential, e.g. low concentrations of noradrenaline. The channels are blocked by sodium nitroprusside and amrinone but are relatively resistant to the organic Ca^{2+} channel antagonists cited above. When potential operated channels are activated in the presence of depolarization produced by an agonist, one cannot exclude a simultaneous activation of receptor operated channels independent of the depolarization.

3. Ca^{2+} channels responsible for resting Ca^{2+} entry: this has to be postulated as the resting Ca^{2+} entry is unaffected by either organic Ca^{2+} channel antagonists or nitroprusside.

In addition to these three Ca^{2+} entry channels, superficially located high and low affinity Ca^{2+} binding sites are present in the sarcolemma(100). Uptake of Ca^{2+} at the high affinity site is preferentially blocked by strontium (Sr^{2+}) allowing a clear qualitative separation of the two sites. On the other hand, aminoglycoside antibiotics (e.g. neomycin, gentamicin) block both high and low affinity Ca^{2+} uptake sites. Whether these agents also block Ca^{2+} entry channels or conversely, whether they block uptake into a surface-bound Ca^{2+} fraction that is subsequently mobilised through channels by stimulating agents is not clear. Uptake of Ca^{2+} into these low and high affinity sites are not blocked by either organic Ca^{2+} channel blockers or sodium nitroprusside.

Evidence was provided by Meisheri et al.(101) in the rabbit aorta for receptor operated and potential operated channels being two sets of channels rather than two different activating mechanisms controlling a single set of channels. It was shown that the Ca^{2+} influx stimulated by having both 80 mmol/l K^+ ions and 10^{-5} mol/l noradrenaline in the tissue bath was approximately equal to the sum of Ca^{2+} influxes when either of the activators was present alone.

Several techniques have been developed in the last decade to measure the shift of Ca^{2+} between the extracellular and intracellular compartments. Van Breemen and co-workers pioneered a method(102,103) in which tissues could be exposed to a variety of agonists in the presence of radioactive Ca^{2+}(^{45}Ca) and subsequently placed in wash out solutions containing La^{3+} ions. The concentration of La^{3+} in these solutions is kept high enough to replace all superficially bound ^{45}Ca and to prevent any further uptake or efflux of ^{45}Ca. The assumption that La^{3+} would replace the extracellular and superficially bound ^{45}Ca and prevent further ^{45}Ca uptake is justified. However, the assumption that La^{3+} would block ^{45}Ca efflux proved to be incorrect(100). Thus modifications such as the use of a higher La^{3+} concentration and a lower temperature during the wash-out period were introduced. The steps of the La^{3+} method as used by Godfraind et al(104) (for the measurement of the uptake of Ca^{2+} into aortic muscle cells stimulated by K^+, and the effect of antagonists on this uptake) are listed below:

1. strips of aorta weighing 6-11 mg were incubated with physiological salt solution containing the antagonist (experimental strips) for 30 minutes, with parallel control strips kept in plain physiological salt solution: the antagonist was present in the buffer solution during the following steps in the experimental strips only.
2. the arterial strips were incubated with physiological salt solution containing ^{45}Ca (1 $\mu Ci\ ml^{-1}$) for 5 minutes and then for another 2 minutes with a depolarizing solution (K^+ 101 mmol/l) also containing ^{45}Ca.

3. preparations were washed for 5 minutes in 500 ml of tris buffer solution (pH 6.8) containing lanthanum chloride (50 mmol/l).
4. the strips were then weighed and ^{45}Ca extracted using perchloric acid and hydrogen peroxide.
5. the radioactivity extracted from the tissue was counted in a liquid scintilation counter.

A modification of this technique can be used to estimate the efflux of ^{45}Ca produced by agonists. Steps of the procedure as used by Godfraind et al(104) (to measure the ^{45}Ca efflux produced by prostaglandin $F_{2\alpha}$ with or without antagonists) are listed below:

1. aortic strips were incubated with physiological salt solution containing ^{45}Ca (3 Ci ml^{-1}) for 120 minutes with the antagonist being present during the last 30 minutes (control strips without the antagonist).
2. tissues were rinsed for 4 minutes in a large volume of non-radioactive physiological salt solution with or without the antagonist.
3. tissues were transferred to a physiological salt solution containing prostaglandin $F_{2\alpha}$ for 3 minutes (with or without the antagonist).
4. the tissues were thereafter treated as in the ^{45}Ca influx experiments - refer above.

A different method utilising a Scatchard plot has been devised to identify and characterise discrete components of ^{45}Ca uptake(100). This type of plot was originally described by Scatchard(105) to describe the binding of small molecules to macromolecules and has been used extensively in radioligand-binding experiments. When utilised for ^{45}Ca uptake studies in smooth muscle the assumptions made are (1) that most of the ^{45}Ca taken up by the cells would be present in bound form and (2) that the extracellular Ca^{2+} concentration is equivalent to the free Ca^{2+} level in the solution. Thus the bound ^{45}Ca and free ^{45}Ca are measured after incubation of the muscle tissue with different extracellular ^{45}Ca concentrations. A plot of bound ^{45}Ca/free ^{45}Ca vs bound ^{45}Ca constitutes the Scatchard plot. This usually results in a curved plot which has at least two linear components in smooth muscle(100): a high affinity binding site for Ca^{2+} apparent at low (0.03 mmol/l) extracellular ^{45}Ca concentrations and a low affinity binding site for Ca^{2+} ions (apparent only at high (1.5-5.0 mmol/l) extracellular ^{45}Ca concentrations. Sr^{2+} in an appropriate concentration can be used to block the high affinity Ca^{2+} ions uptake site while leaving the low affinity uptake sites relatively unaltered. With respect to these results it is also of interest that Sr^{2+} ions can be used as a substitute for Ca^{2+} in supporting a K^{+} induced tension response more than in maintaining a noradrenaline induced tension response in some smooth muscles(100,106).

Although extracelluar Ca^{2+} is an important source of Ca^{2+} for contraction, some vascular smooth muscles respond to agonists with contraction in the absence of extracellular Ca^{2+} ions(29). In the rabbit aorta contraction produced by high concentration of K^{+} declined rapidly in Ca^{2+}-free medium, whereas those to noradrenaline and histamine persisted with less reduction(107). Extracellular La^{3+} too has a lesser inhibitory effect on contraction produced by noradrenaline than on contractions produced by K^{+} depolarization lending further support to the relese of intracellular Ca^{2+} during noradrenaline induced contractions(29). Thus an intracellular pool probably provides the Ca^{2+} for part of the contraction produced by noradrenaline and histamine.

However, the contractions induced by these agonists in the absence of extracellular Ca^{2+} are reduced to one or at the most, two contractions suggesting that the intracellular Ca^{2+} pool is a limited one because it must be refilled from the outside(102). This intracellular pool takes up Ca^{2+} from the extracellular medium by a process sensitive to lanthanum blockade but not to Ca^{2+} antagonists such as methoxyverapamil in the rabbit aorta(108).

Deth and Casteels(109) in some elegant experiments in the rabbit aorta, investigated the effects of different agonists on the intracellular pools of Ca^{2+} involved in contractions as described above. As the method used in these experiments is another technique used in the measurement of Ca^{2+} fluxes in smooth muscle the steps of the procedure as used by Deth and Casteels(109) are listed below:

1. tissue strips weighing approximately 15 mg were incubated for 3 hours in 10 ml of physiological salt solution containing ^{45}Ca, 1.5 mmol/l (5×10^6 cpm/ml).
2. the tissues were then rinsed for 5 seconds in a large volume of physiological salt solution to remove the adherent loading solution.
3. the tissues were transferred at 5- or 10-minute intervals through a series of previously washed scintilation vials containing 5 ml of physiological salt solution with a Ca^{2+} concentration of 1.5 mmol/l. Oxygen was bubbled through the solution to maintain oxygenation of the tissue. Deth and Van Breemen used Ca-EGTA in this efflux medium to minimize backflux of ^{45}Ca during the efflux(110).

4. noradrenaline or caffeine or 2,4-dinitrophenol were introduced into the physiological salt solution in the scintilation vials at various times after the commencement of the efflux measurements.
5. the tissue strips were dried and weighed at the end of experiments and the ^{45}Ca remaining in the tissue extracted by digestion.

The efflux of ^{45}Ca measured in this fashion follows an exponential pattern. Addition of noradrenaline or caffeine resulted in an immediate increase of the efflux which reached a maximum within 10 minutes. In contrast the response to 2, 4-dintrophenol (DNP) appeared more slowly reaching a maximum only after 20 minutes. A similar difference in the time course of action was noted on tension development in rabbit aortic rings in separate studies carried out simultaneously. Further, a plot of the amount of ^{45}Ca efflux (estimated from the area under the efflux curve above the control) and the concentration of noradrenaline showed a good correlation with a plot of tension generated against the concentration of noradrenaline. The intracellular Ca^{2+} pools utilised by noradrenaline and caffeine appeared to be a single pool as under extracellular Ca^{2}-free conditions, a prior transient caffeine exposure eliminated the ^{45}Ca efflux produced during a subsequent exposure to noradrenaline. Estimates of the ^{45}Ca pool sizes and the rates of exchange for the three agonists suggested that the noradrenaline and caffeine probably shared a single pool while DNP appeared to ^{45}Ca from a separate pool. K^{+} depolarization utilised during the ^{45}Ca loading procedure to increase cellular uptake of ^{45}Ca also showed a differential effect on the three agonist induced effects. The ^{45}Ca efflux produced by DNP was increased three- to four-fold by this manoueuvre while, rather surprisingly, the efflux produced by caffeine and noradrenaline were reduced. The above study leads one to conclude that at least two different pools of intracellular Ca are involved in the contractions produced by the three agonists in the rabbit aortic muscle. Other studies too suggest at least two intracellular pools: a low affinity (rapid release) pool responsive to noradrenaline, histamine, acetylcholine, caffeine, and a high affinity (slow release) pool somewhat sensitive to these agents but more specifically sensitive to DNP.(30). A separate study on the rabbit aorta by Deth and Van Breemen(110) demonstrated that although noradrenaline produced an increase in ^{45}Ca efflux this could not be reproduced with a second exposure to the drug. Thus the ^{45}Ca released from the intracellular store by noradrenaline is unlikely to be taken back into the same store.

Intracellular calcium stores

Although the role of intracellular Ca^{2+} pools in providing calcium for contractions produced by some agonists is accepted, there is considerable debate as to the exact source(s) of this intracellular Ca^{2+}. The possible sites mentioned are the sarcoplasmic reticulum, the mitochondria and the sarcolemma. The sarcoplasmic reticulum is developed to a varying extent in different smooth muscle cells, constituting approximately 2.0-7.5 per cent of the cell volume. The higher values found in the large elastic arteries include a significant proportions of rough endoplasmic reticulum whose Ca^{2+} storage capacity may not be as much as that of the smooth reticulum. However, even if the sarcoplasmic reticulum constituted only 2 per cent of the cell volume, assuming that like the reticulum of striated muscle, it contains 25 mmol/l of Ca^{2+}, a complete release of the latter would be more than sufficient to produce maximum activation of a smooth muscle cell(81). The volume of sarcoplasmic reticulum in different smooth muscles correlate well with their ability to contract in the absence of extracellular Ca^{2+} ions(32,33). As these measurements did not distinguish between rough and smooth endoplasmic reticulum in the different smooth muscles and as the permeability of the sarcolemma is known to differ between different smooth muscle it is possible that this correlation is spurious(81). Surface couplings in smooth muscle are specialised regions similar to those in cardiac muscle where the sarcoplasmic reticulum is separated from the surface membrane by only a 10-12 nm space traversed by periodic electron opaque processes. These have been suggested as sites where depolarization spreading along the sarcolemma may trigger the release of Ca^{2+} from the reticulum. Although sarcoplasmic reticulum is a possible candidate as a Ca^{2+} source, no definite structural evidence for the actual release of Ca^{2+} from the reticulum is available at present(111).

Isolated mitochondria from vascular smooth muscle are capable of accumulating Ca^{2+} and other divalent cations such as Ba^{2+} and Sr^{2+}(81). This has been demonstrated by electron probe analysis and electron microscopy. The apparent Km for the Ca^{2+} uptake by mitochondria was 17 μmol(39). This high Km of isolated mitochondria for Ca^{2+} suggests that mitochondria are unlikely to regulate cytosolic free Ca^{2+} concentration during the normal contraction-relaxation cycle. At a intracellular free Ca^{2+} concentration of 1 μmol/l (which is believed to produce about 50-80 per cent of the maximum contraction in smooth muscle) the effect of the mitochondrial transport system would be minimal.

An alternative possibility is that the inner surface of the plasma membrane is the principal storage site from which Ca^{2+} ions are released during contraction. Suzuki, Sugi and co-workers(112,113) demonstrated (using electron microscopy) that in cells treated with potassium oxalate, the intracellular Ca^{2+} was localised to the peripheral cytoplasm adjacent to the plasma membrane in the relaxed state: following drug induced contraction in the absence of external Ca^{2+}, the Ca^{2+} stain was found diffusely throughout the cytoplasm. This suggested that the Ca^{2+} bound to the inner surface of the sarcolemma was released into the sarcoplasm during the contraction. The role played by the surface vesicles or caveolae in possible Ca^{2+} regulation by the sarcolemma is not clear at the present time.

Calcium sequestration during relaxation

From the foregoing discussion it is apparent that there is considerable controversy about the source of Ca^{2+} for activation in smooth muscle. However, there is an even bigger debate about how the free Ca^{2+} ions are removed from the sarcoplasm during relaxation in smooth muscle. Relaxation in smooth muscle as in striated muscle is brought about by a lowering of the sarcoplasmic free Ca^{2+} leading in turn to a decreased actomyosin-ATPase activity and a detachment of the cross-bridges. This lowering of the sarcoplasmic Ca^{2+} can be brought about by several processes similar to those observed in cardiac muscle. First, at the sarcolemna the inward Ca^{2+} current that occurs during depolarization is self-limiting(30). This is brought about by an increase in permeability to K^+, which follows the rise in Ca^{2+} and Na^+ permeability, in smooth muscle with action potentials, and occurs simultaneously in smooth muscle which show graded depolarizations only. The rise in intracellular Ca^{2+} may itself be the trigger for the rise in K^+ permeability which results in an outward 'rectifying' K^+ current. The resulting repolarization reduces membrane permeability to Ca^{2+} to its resting level. The termination of the inward Ca^{2+} current by itself, however, does not lead to relaxation as the Ca^{2+} that accumulated in the sarcoplasm during the inward current has to be removed from the vicinity of the contractile proteins. This Ca^{2+} is either extruded out of the cells at the sarcolemma or taken up into intracellular storage sites.

Transport of Ca^{2+} ions out of the cells is an energy consuming process as it involves a transfer against a large electrochemical gradient(114). There are two principal mechanisms through which Ca^{2+} is believed to be transported out of the cells: (1) the Na^+/Ca^{2+} exhange system and (2) the Mg-ATPase dependent extrusion system. The features of the Na^+/Ca^+ exchange system can be summarised as follows(115):

1. Na^+ ions move down its electrochemical gradient from the extracellular fluid to inside the cell.
2. coupled to this inward movement of Na^+ ions, Ca^{2+} ions are extruded from the cell.
3. the energy liberated by the inward movement of three Na^+ ions is required to move one Ca^{2+} ion out of the cell.
4. ATP may modify the kinetics but is not a primary source of energy for the transport system: the energy being provided by the electrochemical gradient for Na^+ ions.
5. for the pump to function the electrochemical gradient of Na^+ has to be maintained via the Na^+/K^+ membrane pump. ATP is of course, utilised for this.
6. the transport system can also move the ions in the reverse direction (i.e., Na^+ outwards and Ca^{2+} inwards) across the membrane when the gradients are suitably altered, because of the competition between Na^+ and Ca^{2+} at sites on both sides of the membrane.

The evidence for the existence of the Na^+/Ca^{2+} pump in smooth muscle is indirect, unlike the situation in the squid axon where Na^+

dependent ^{45}Ca fluxes have been shown(114). In vascular smooth muscle lowering of external Na^+, for instance by replacement with Li^+ leads to an increase in tension. Further, introduction of ouabain, the Na^+/K^+-ATPase inhibitor, often leads to contraction in vascular smooth muscle associated with an elevation of intracellular Na^+ concentration. Both these manoeuvres lead to the loss of the Na^+ gradient which would inhibit the Na^+/Ca^{2+} pump as proposed. Re-establishing the Na^+ gradient by re-introduction of the Na^+ or removing ouabain in the above experiments leads to a relaxation. Although the evidence is strong that Na^+ plays some role in the control of intracellular Ca^{2+}, it is nevertheless quite clear that an inwardly directed Na^+ gradient is not essential for the tissues to be able to regulate intracellular Ca^{2+}(83). Brading(116) demonstrated that smooth muscle can not only remain in the relaxed state but also contract and relax again to stimuli in the absence of a Na^+ gradient, although the response may be qualitatively different from the control state. Other workers too have found similar results(114). Because of these inconsistencies, ATP-dependent extrusion of Ca^{2+} ions has been suggested as an alternative to the Na^+/Ca^{2+} transport system. This Ca^{2+} pump is believed to be similar to that described in red blood cells by Schatzmann(117). Again, direct evidence for the existence of such a Ca^{2+} pump in vascular smooth muscle is lacking(87). It is possible that both systems described above are present in smooth muscle. It has also been suggested that the Ca^{2+}/ATPase system may be located on the sarcoplasmic reticulum membrane and the Na^+/Ca^{2+} exchange system between the sarcoplasmic reticulum and the extracellular fluid: the Ca^{2+} from the sarcoplasm is first taken into the reticulum via the Ca^{2+}/ATPase system and subsequently extruded into the extracellular fluid via the Na^+/Ca^{2+} exchange system.

Sarcoplasmic reticulum, mitochondria, nucleus and the inner surface of the sarcolemma are the possible intracellular storage sites for Ca^{2+} ions. The majority of the studies designed to investigate the possible Ca^{2+} storage sites have used differential centrifugation of smooth muscle homogenates to isolate subcellular fractions that have been proposed to be potential sinks and sources of activator calcium. Mitochondria are isolated by sedimentation between 9500 and 15,000 g and a microsomal fraction obtained at 40,000-15,000 g(29). The latter fraction, however, contains a mixture of sarcoplasmic reticulum and sarcolemmal fragments. A fundamental problem in identifying specific sites of calcium accumulation in smooth muscle has been the fact that it has been difficult to isolate either pure or enriched fractions of sarcoplasmic reticulum and sarcolemma. The greatest difficulty has been the reliable separation of reticulum fractions from sarcolemmal fractions. This subject has been recently reviewed by Allen and Bukoski(94). Mitochondria have been isolated in sufficiently pure form during the last few years allowing the characterisation of the Ca^{2+} accumulation by these organelles. These studies suggest the presence of an energy-dependent mitochondrial Ca^{2+} transport system(81). As mentioned before, this system has a low affinity but a high capacity, thus it is unlikely that mitochondria play a major role in the sequestration of Ca^{2+} ions during relaxation in smooth muscle. However, insufficient data is available at present to definitely exclude a greater role for mitochondria in a sub-type of smooth muscle such as the tonic variety(81).

Microsomal fractions of smooth muscle have been shown to accumulate Ca^{2+} by an ATP-dependent system. However, the difficulty of separation of reticular fragments from the sarcolemmal fragments have made it difficult to draw a firm conclusion as to the membrane fragment responsible for the transport system(94). The requirements for the uptake of Ca^{2+} by microsomal fractions in smooth muscle are similar to those for striated muscle with both ATP and Mg^{2+} ions being required. This uptake depends on the temperature, being higher at 37°C than at 0°C: optimum pH values reported have been 7.4 and 7.8(29). Although sarcoplasmic reticulum is a likely site for the Ca^{2+} sequestration during relaxation its role has not been definitely established. It has been postulated that the inner sarcolemma may function as the prinipal intracellular release site for Ca^{2+} during contraction with the Ca^{2+} being subsequently sequestered by the sarcoplasmic reticulum(110). Current evidence suggests that Ca^{2+} released from intracellular stores during contractions produced by some agonists in a Ca^{2+}-free medium is probably not sequestered back to the same store: only one or at the most two, contractions can be induced by these agonists in a Ca^{2+}-free medium; in ^{45}Ca-efflux experiments ^{45}Ca released during the first exposure to noradrenaline does not return to the release sites, as evidenced by the lack of the increased efflux upon a second exposure to the agonist. It could be further postulated that the calcium sequestered in the sarcoplasmic reticulum in smooth muscle is extruded into the extracellular space by the Na^{+}/Ca^{2+} exchange sytems and then returns to the release sites at the inner sarcolemma.

Role of cyclic nucleotides in the regulation of contraction/relaxation

There has been considerable debate during recent years about the role played by the cyclic nucleotides, cyclic adenosine 3':5'-monophosphate (cyclic-AMP) and cyclic guanosine 3':5' monophosphate (cyclic-GMP) in the regulation of contraction. This subject has been reviewed by Kramer and Hardman recently(118,119). Cyclic-AMP is produced by the action of the enzyme adenylate cyclase on ATP. Beta adrenergic stimulation is known to cause an activation of the adenylate cyclase system with the production of cyclic-AMP. The degradation of cyclic nucleotides including cyclic-AMP is carried out by cyclic nuleotide phosphodiesterases. These enzymes or family of isoenzymes, catalyses the hydrolysis of the 3' bond of the 3':5' cyclic phosphate to give the 5'-monophosphate. Cyclic-AMP exerts most of its effect through the activation of protein kinases located in the cells(119). The protein kinases are composed of two sub-units: the catalytic sub-unit and the regulatory sub-unit. The latter inhibits the action of the former as long as it remains bound to it. Cyclic-AMP activates the enzyme by combining with the regulatory sub-unit, thereby causing its dissociation from the catalytic sub-unit of the enzyme. The activated protein kinase can thereupon phosphorylate a variety of substrate proteins by transfer of a gamma-phosphate group from ATP, e.g. glycogen synthase, phosphorylase kinase, etc. One such substrate protein phosphorylated by the cyclic-AMP dependent protein kinase, is the enzyme myosin light chain kinase. Phosphorylation of the light chain kinase reduces its affinity for calmodulin leading to decreased phosphorylation of myosin light chains and thus a decrease in contraction (refer above, under Phosphorylation theory for details). This is believed to be one mechanism by which cyclic-AMP induces relaxation in smooth muscle(120). The other mechanism proposed has been an enhancement of sequestration of Ca^{2+} by the sarcoplasmic reticulum during relaxation in smooth muscle. Incubation of vascular smooth muscle microsomes with cyclic-AMP, has been shown to accelerate the energy-dependent Ca^{2+} uptake by the microsomal frations(121,122). These microsomal fractions may contain fragments from both the sarcoplasmic reticulum and the plasma membrane as mentioned before. It is possible that cyclic-AMP activates the Ca^{2+}-ATPase in either membrane which, in turn, could increase Ca^{2+} uptake into the reticulum or the extrusion of Ca^{2+} across the plasma membrane or both. It has been suggested that cyclic-AMP is a mediator of the relaxation produced by certain drugs. The evidence for this cyclic-AMP hypothesis can be summarised as follows(29,30,123).

1. β-adrenergic agonists cause a relaxation of smooth muscle accompanied by an increase in cyclic-AMP levels.
2. A quantitative and a temporal correlation between the elevation of cyclic-AMP and the relaxation has been shown with isoprenaline(124)
3. β-blocking agents reduce both the generation of cyclic-AMP and the relaxation produced by β-agonists.
4. Relaxatory response to β-adrenergic stimulation is potentiated by agents that inhibit the enzyme phosphodiesterase which breaks down cyclic-AMP.
5. Inhibitors of phosphodiesterase themselves cause relaxation of smooth muscle with an increase in cyclic-AMP levels, e.g. papavarine. This action is complicated by the fact that these agents have actions other than the inhibition of the phosphodiesterase activity. Some of these drugs block the uptake and facilitate the release of Ca^{2+} from the sarcoplasmic reticulum. Theophylline, in addition, antagonises the effect of the vasodilator adenosine at the P_1-purinoceptor. Thus, some phosphodiesterase inhibitors may actually produce smooth muscle contraction in spite of elevating cyclic-AMP levels.
6. Cyclic-AMP and dibutyryl cyclic-AMP (the latter has a higher tissue permeability) have been shown to induce relaxation in smooth muscle on their own.

7. As mentioned above, two possible mechanisms of action for cyclic AMP induced relaxation has been demonstrated in smooth muscle.
8. Other drugs which produce relaxation in smooth muscle also increase cyclic-AMP levels, eg. prostacyclin, adenosine(123).

Although the above evidence appears substantial, some doubts remain as the results have not been consistent from different centres(125). For instance, during a K^+ depolarization, propranolol, while blocking the isoprenaline-induced rise in cyclic-AMP, leaves the relaxation produced by the isoprenaline intact(126). In demonstrating that cyclic nucelotides have a function in a cellular response, it is necessary to show not only that cyclic nucleotides are altered by the same agents that evoke the physiological response, but also that these alterations are correlated quantitatively and temporally. Unfortunately, the majority of the studies in support of a role for cyclic-AMP in smooth muscle relaxation do not fulfill this requirement. Cyclic-AMP levels have been often measured at only one time point of contraction or relaxation, at only one concentration of the agonist or under incubation conditions when the tissue was not under a mechanical tension(118). Interpretation of the investigations carried out to demonstrate the direct relaxant effects of cyclic-AMP and dibutyryl cyclic-AMP have proved difficult as questions concerning the ability of the nucleotides to reach the site of action, and questions concerning the effects of hydrolysis products or impurities have been seldom dealt with, in these studies. Thus, controls using adenosine, butyrate, other nucleotides and possible contaminants have not been tested adequately(118). This precaution is particularly important as high concentrations (100-1000 fold higher than the physiological level of cyclic-AMP and dibutyryl cyclic-AMP have often been employed in these studies. A further complication arises because cyclic nucleotide derivatives are also capable of inhibiting phosphodiesterase activity. Thus a derivative of cyclic AMP might cause elevation of endogenous cyclic-GMP and vice versa. With regard to the studies using phosphodiesterase inhibitors, again, no clear demonstration of the relationship between the relaxation and the time-course and magnitude of the changes in cyclic-AMP levels has been carried out. Cyclic-AMP is believed to increase the uptake of Ca^{2+} by microsomal fractions of smooth muscle via a protein kinase. However, studies by different investigators have resulted in inconsistent findings: some showing phoshorylation and uptake of Ca^{2+}; others showing phosphorylation with no Ca^{2+} binding or vice versa; and rarely no effect on phosphorylation or uptake of Ca^{2+}(118).

With regard to the role of cyclic-GMP in smooth muscle contraction/relaxation, less information is available at the present time. Early studies reported an elevation of cyclic-GMP levels accompanying contractions in smooth muscle produced by a variety of agonists including prostaglandin $F_{2\alpha}$, acetylcholine and alpha-adrenergic agents(118). Further, it was shown that Ca^{2+} was necessary for the above effect. On the basis of these studies, cyclic-GMP was considered as a mediator of smooth muscle contractions. However, subsequent studies demonstrated a clear dissociation between the contraction produced and the cyclic-GMP levels. In addition, nitroglycerine, nitroprusside and several nitrogen-containing compounds have been shown to produce large increases in cyclic-GMP (but not of cyclic-AMP) with an accompanying relaxation(127,128). These agents do not generally require Ca^{2+} for their effects on cyclic-GMP levels. Thus during the last few years cyclic-GMP has been investigated as a mediator of smooth muscle relaxation. Kukovetz et al studied the effect of acetylcholine in the absence and presence of M&B 22948, a potent inhibitor of the hydrolysis of cyclic-GMP(123). Acetylcholine, on its own caused a contraction of the arterial strip accompanied by an increase in cyclic- GMP. In the presence of M&B 22948 the dose response curve to acetylcholine was shifted to the right accompanied by a further increase in cyclic-GMP levels. Thus, a higher level of cyclic-GMP was associated with a lesser contractile response. This study casts serious doubts on any role for cyclic GMP in the contractile effects produced by acetylcholine; it suggests a possible role on a relaxatory effect for cyclic-GMP. Further support for the latter is provided by the fact that methylene blue, while attenuating the increase of cyclic-GMP produced by acetylcholine in the bovine coronary artery, increased the contractile response produced by the drug(123). Although an interesting association between relaxation and cycic-GMP in smooth muscle has been demonstrated, no definite proof for a cause and effect relationship is available at the present time.

In summary, it could be said that it appears likely that cyclic-AMP is involved in the relaxation in smooth muscle produced by at least the beta-adrenergic agonists. However, it has not been conclusively shown that this cyclic nuceotide is an absolute necessity in the mediation of the relaxation, although the association is strong. With regards to cyclic-GMP, much more work has to be done before any conclusion can be arrived at. Further, it is not likely that all smooth muscle relaxing drugs would act through a cyclic nucleotide mechanism.

ADRENERGIC NEUROEFFECTOR INTERACTION

Several excellent reviews on the subject have been published during recent years (15,73,129). Almost all blood vessels are innervated with post-ganglionic sympathetic nerves although the density of innervation varies widely. As in other adrenergically innervated tissues the ability to synthesise, store, and release noradrenaline resides in the adrenergic nerve terminals found in the walls of the blood vessels.

Transmitter synthesis and storage (Fig. 10)

Noradrenaline and adrenaline are synthesised from the precursor amino acids, phenylalanine and tyrosine(130). The sequence of events has been confirmed and the enzymes involved identified and characterised. Tyrosine, which is generally available in the body fluids is taken up by the nerve terminals and converted to 3,4-dihydroxyphenylalanine or DOPA by the enzyme tyrosine hydroxylase. This enzyme, present in the neuroplasm, regulates the biosynthesis of the catecholamines, as the hydroxylation of tyrosine is slower than the subsequent steps. Several physiological mechanisms are present to modulate the activity of this enzyme and thereby regulate noradrenaline synthesis. In the next step in the synthetic pathway DOPA is decarboxylated to form dopamine in the neuroplasm. The dopamine is then taken up by the storage vesicles contained within the adrenergic nerve endings, where it is converted to noradrenaline by the enzyme dopamine-β-hydroxylase. As dopamine-β-hydroxylase is not present in the neuroplasm, dopamine must be taken up into the vesicle before noradrenaline could be formed. In the adrenal medulla, most of the noradrenaline leaves the granules and is methylated in the cytoplasm to adrenaline and then re-enters a different group of intracellular storage vesicles, where it is stored until released. In peripheral sympathetic nerve endings the enzyme phenylethanolamine N-methyltransferase(PNMT) is absent and the principal transmitter is noradrenaline. However, about five per cent of the released transmitter at these nerve endings may

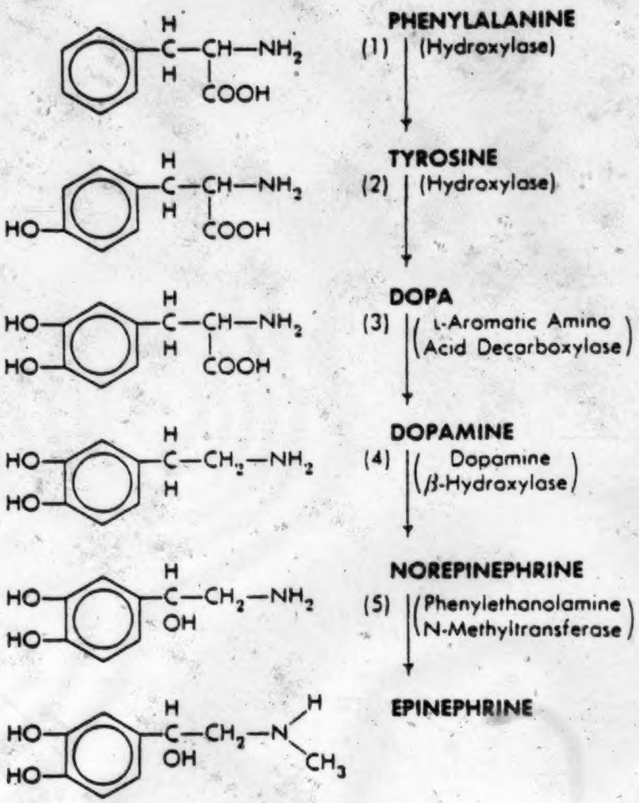

PHENYLALANINE
(1) (Hydroxylase)
TYROSINE
(2) (Hydroxylase)
DOPA
(3) (L-Aromatic Amino Acid Decarboxylase)
DOPAMINE
(4) (Dopamine β-Hydroxylase)
NOREPINEPHRINE
(5) (Phenylethanolamine N-Methyltransferase)
EPINEPHRINE

Figure 10. Summary of the steps in the synthesis of noradrenaline and adrenaline in the sympathetic nervous system. Steps 1-3 take place in the neuroplasm and step 4 in the adrenergic storage vesicles subsequent to transport of dopamine into the storage vesicles from the neuroplasm.

consist of adrenaline; this probably reflects adrenaline from the circulation that is taken up into the nerve endings (when plasma concentration of adrenaline is raised) and released subsequently together with noradrenaline.

It should be noted that some of the enzymes involved in the pathway of catecholamine synthesis are quite non-specific, capable of acting upon many other endogenous substances as well as certain drugs (130). For instance 5-hydroxytryptamine (5-HT, serotonin), tyramine and histamine can be produced by L-aromatic acid decarboxylase (or DOPA decarboxylase) from their corresponding amino acids. Tyramine, in turn can be oxidised to octopamine, the phenol analogue of noradrenaline; while octopamine is present only in small amounts in mammals, it is probably the major adrenergic neurotransmitter in certain invertebrate species. The antihypertensive drug α-methyldopa is also metabolised by this pathway forming α-methyldopamine and subsequently the "false transmitter" α-methylnoradrenaline. The enzymes involved in the pathway are probably synthesised in the perikaryonal cell bodies of the adrenergic neurones and then transported along the axons to their terminals. A slow transport system (1 to 3 mm per day) and a fast (1 to 10 mm per hour) system have been documented. Tyrosine hydroxylase is believed to utilise the slower transport system and dopamine-β-hydroxylase the faster system. Microtubular system present in the axons may play a role in this fast tranpsort system (130,131).

Several mechanisms regulate the synthesis of noradrenaline via modulation of the tyrosine hydroxylase activity either on the short-term or on a long-term basis(73). First, the end product noradrenaline modulates the activity of the enzyme by feed-back inhibition. This same mechanism may account for increased activity of the enzyme with accelerated synthesis of noradrenaline during nerve stimulation. The total neuronal content of noradrenaline need not necessarily be decreased for the enzyme to be activated: thus a specific compartment of noradrenaline in the cytosol probably participates in this regulating mechanism. In addition, the influx of Ca^{2+} that takes place during nerve stimulation may also induce the enzyme. This may help to accelerate the synthesis of noradrenaline during continued sympathetic nerve stimulation. Trans-synaptic mechanisms may also inhibit (via pre-synaptic α_2 receptors) or activate (via pre-synaptic β-receptors) the enzyme. Other compounds such as angiotensin, prostaglandin and acetylcholine too may regulate the tyrosine hydroxylase activity in a similar manner(130). Acute (short-term) regulation probably involves allosteric effectors that alter the affinity of the enzyme for co-factors, substrate or end-product inhibition. Long-term regulation occurs by other mechanisms such as alteration in the synthesis of the enzyme and covalent modification of the structure of the enzyme. The effect of these long-term modulations usually takes a few days to develop, but they can persist for several weeks; the delay probably reflects the time required for transport of newly synthesised enzyme from cell body to the nerve terminal.

Noradrenaline content of the walls of blood vessels ranges from 0.08 μg/g wet weight in the rat aorta to nearly 10 μg/g wet weight in the guinea pig uterine artery(73). Although such measurements may provide a rough indication of the density of adrenergic innervation they can be misleading, especially when expressed as a function of tissue weight. Noradrenaline present in adrenergic nerve endings is stored in granular vesicles (also called storage vesicles or dense-core vesicles) with the vesicular membrane providing protection from the intraneuronal enzyme monoamine oxidase. The noradrenaline in the vesicles is complexed with ATP and proteins, of which chromagranin A is the most important. ATP and noradrenaline are present in a molecular ratio of 1:4(130). Dopamine-β-hydroxylase is also present in the vesicles. The vesicles, at least in their initial state, are believed to be synthesised in the cell body and tranpsorted down the axon.

Two types of storage vesicles can be identified by electron-microscopy in adrenergic nerve endings; small dense-core vesicles, 30-60 nm in diameter and the large dense-core vesicles, 60-120 nm in diameter(132). The large dense-core vesicle can constitute 5-50 per cent and the small dense-core vesicle 50-95 per cent of the vesicle population in an adrenergic nerve terminal, although the latter predominate in most cases. In general, the larger animals seem to possess higher numbers of large dense-core vesicles in their adrenergic nerve endings. It should be understood that a much smaller percentage of large dense-core vesicles may provide a large core volume (i.e. storage capacity for noradrenaline) at the nerve endings. Thus, in a nerve ending containing 10-15 per cent large vesicles and 85-90 per cent small vesicles, the core volume for the two types of vesicles appear to be equal. In man, the large vesicles may comprise up to 80-90 per cent of the total storage capacity in certain adrenergic varicosities(132). Small vesicles are believed to contain up to a 1000 molecules of noradrenaline per vesicle while a large vesicle may contain up to 16,000 molecules. Two adrenergic vesicle populations can also be distinguished by separation using sucrose density gradients(133). Although the low density peak of noradrenaline is enriched in small dense-core vesicles and the high density peak is thought to correspond to the large dense-core vesicles, the correlation between electron-microscopy and the biochemical methods is by no means certain(73).

Transmitter release

The stored noradrenaline may exit from the nerve endings via the neuroplasm or directly by exocytosis(15). In the resting state, a small amount of the stored noradrenaline diffuses into the neuroplasm continuously through the vesicular membrane. Most of it is deaminated by the enzyme monamine oxidase (MAO) to 3,4 dihyhydroxyphenylglycol (DOPEG), but a small fraction escapes into the synaptic cleft as intact noradrenaline together with DOPEG and the other metabolites formed within the nerve ending. The minute amounts of intact noradrenaline leaking out produce miniature excitatory junction potentials which can be recorded. However, the amounts released are usually not great enough to produce any active tension in most arteries. Drugs such as tyramine, amphetamine, ephedrine and guanethidine are taken up into the adrenergic storage vesicle and lead to the displacement of the noradrenaline into the neuroplasm. They probably exert their effect by having a greater affinity for the storage proteins than noradrenaline(15). As with the spontaneously leaking transmitter, part of the displaced noradrenaline is deaminated to DOPEG but a sizeable fraction is released as intact noradrenaline to produce effects on the smooth muscle. Pharmacological displacement of the transmitter, unlike the exocytotic process, is not dependent on an increase in neuroplasmic free Ca^{2+} concentration. As the indirectly acting sympathomimetic drugs have to be taken up into the nerve terminal by $uptake_1$ (see below) before they could exert their effects, their action is blocked by inhibitors of uptake$_1$. On the other hand, inhibitors of MAO, by preventing the deamination of the displaced noradrenaline, potentiate the effects of indirect sympathomimetic agents.

During increased sympathetic activity, propagated action potentials spreading along post-ganglionic sympathetic nerves result in the release of noradrenaline by exocytosis at the adrenergic nerve terminals. At the nerve terminal the inward current of the action potential is carried by Na^+ and Ca^{2+} ions. Increase in the neuroplasmic Ca^{2+} results in the exocytosis of the storage vesicles with release of noradrenaline and other vesicular contents (such as the enzyme dopamine-β-hydroxylase) into the synaptic cleft. In isolated smooth muscle, transmural nerve stimulation induces release of noradrenaline by exocytosis, by excitation of the intramural nerves contained in the isolated muscle with induction of action potentials(2). High K^+ solutions act on isolated smooth muscle containing adrenergic nerves, leading to depolarization of the nerve endings with release of the stored noradrenaline by exocytosis; as with an action potential an inward Ca^{2+} current accompanies the direct depolarization produced by the K^+ ions triggering the release of the transmitter. However, it should be noted that K^+ may exert a number of other actions on isolated smooth muscle(134). The action potential induced release of noradrenaline is blocked by tetrodotoxin, the fast Na^+ channel inhibitor(135). However, the release produced by high K^+ is not inhibited by tetrodotoxin as the K^+ causes a direct depolarization rather than a depolarization via the induction of action potentials(136). The Ca^{2+} current induced by high K^+ depolarization at the nerve terminal can be inhibited by the Ca^{2+}

channel blockers verapamil, prenylamine and D600 although at concentrations higher than the usual therapeutic ranges(137). This results in a decrease in transmitter release.

During exocytotic release of the transmitter, only a small (up to 5 per cent) percentage of vesicles within a varicosity release their contents. Further, any single varicosity does not respond to every stimulus by releasing transmitters; thus the release is intermittent and believed to occur once every 7-8 action potentials(132). It is not known whether the intermittency of release is because the varicosity is not always invaded by the action potential, or because it is incapable of (or refractory to) transmitter release for a finite period of time following release. Noradrenaline is released from both large dense-core and small dense-core vesicles during exocytosis. The release of transmitter by exocytosis from the two types of vesicles has been observed in electron-micrographs taken following superfusion fixation of fine smooth muscle strips during transmural nerve stimulation(132). However, there is a debate at the present time as to whether each vesicle releases all its contents (total content release) or only part of its contents with exocytosis (partial content release)(73). Vesicle re-cycling would be a necessary occurrence with the latter theory although it could take place even after total content release and thus does not consititute evidence against the former. There is evidence that vesicle re-cycling occurs at nerve terminals including adrenergic synapses during transmitter release(138). Extracellular markers such as horseradish peroxidase have been demonstrated inside vesicles following transmitter release induced by transmural nerve stimulation.

Basbaum and Heuser in a study in the mouse vas deferens(139) demonstrated that the adrenergic varicosities contained approximately 93 per cent small vesicles and 7 per cent large vesicles; 73 per cent of the former and 95 per cent of the latter contained electron-dense cores. The core sizes varied from 50-80 per cent of the vesicle diameter in the small dense core vesicles and were more uniform and typically measured 80 per cent in the large dense-core vesicles. In muscle segments fixed immediately following transmural nerve stimulation a 60 per cent reduction in the small vesicles with no change in the large vesicle number was observed. Moreover, in the small vesicles that remained, only 31 per cent contained dense-cores and in most of these the core size was smaller compared to the pre-stimulation period. This decrease in the small vesicle population was accompanied by a significant increase in the mean perimeter of the varicosities. The small vesicle numbers, and the mean perimeter of the varicosities returned to control values after 2 hours. By this time the core sizes had also recovered to mid-way between control and immediate post stimulation values. In this study, the uptake$_1$ (see below) inhibitor desmethylimipramine in a concentration of 3×10^{-5} mol/l did not significantly affect the recovery of the small vesicle numbers or their dense-core sizes. The extracellular marker horseradish peroxidese was present in small dense-core vesicles following transmural nerve stimulation whereas it did not enter the variosities in unstimulated muscles even after 80 minutes incubation. The following conclusions can be arrived at from this study. (1) Small dense-core vesicles are involved in transmitter release in this preparation. (2) Part of the vesicular membrane appears to be incorporated into the cell membrane of the varicosity during transmitter release, as evidenced by the increase in the mean perimeter of the varicosity following the transmural nerve stimulation. (3) However, vesicle recycling (or the formation of entirely new vesicles) at the cell membrane of the varicosity, appears to take place during transmitter release, as evidenced by the presence of horseradish perioxidase in the vesicles following the nerve stimulation . (4) Formation of new vesicles at the synaptic membrane appears to continue following the stimulation period: the mean perimeter of the varicosities and the vesicular number returning to control values during a two hour post-stimulation period. (5) Replenishment of transmitter stores in the vesicles is not dependent on re-uptake of noradrenaline released during nerve stimulation.

Although only small dense-core vesicles were observed to be released in the above study, others believe that large-dense core vesicles are also involved in transmitter release. Nelson and Molinoff(133) demonstrated that the indirectly acting sympathomimetic agent tyramine caused equal depletion of noradrenaline from both high and low density fractions isolated from sucrose density gradients. Further, some believe that the small dense-core vesicles are formed at the synaptic membrane subsequent to exocytotic release from the large dense-core vesicles(132,140). An alternative hypothesis is that direct splitting of the large dense-core vesicles within the varicosity gives rise to the smaller vesicles. However, there is very little evidence for the latter hypothesis at the present time(132). The former hypothesis is supported by the fact that very little dopamine-β-hydroxylase and other soluble proteins (which would have been released at the time of exocytosis) are present in the small dense core vesicles. This would explain the controversy in the literature over the correlation, or lack of it, between the released catcholamine levels and the dopamine-β-hydroxylase levels during sympathetic stimulation. As both populations of vesicles contain the transmitter and only one type contains the enzyme, the ratio of the released transmitter to the released enzyme would depend on the relative proportions of the two populations of vesicles; this differs from tissue to tissue as well as with the functional status even in a single tissue as explained above. The hypothesis could also explain how antigenic activity might be retained between the large and small dense-core vesicles. At the present time, the exact origin of small dense-core vesicles is still uncertain. Large dense-core vesicles, on the other hand, are known to be formed in the neuronal cell body and transported down the axon to the nerve terminal. During this passage the vesicular content of noradrenaline increases while the dopamine-β-hydroxylase activity and the ATP content of the vesicle remain unchanged(141).

Another controversial area in neurotransmission in smooth muscle is the different sub-cellular compartments involved in transmitter release: these sub-cellular compartments may be different types of vesicles (e.g. large and small dense-core), a sub-population of a single type of vesicle (e.g. vesicles which have gone through a cycle of exo- and endocytosis being refilled with neurotransmitter and preferentially released), or different pools of the transmiotter within a vesicle. Preferential release of newly synthesised transmitter was demonstrated in various cholinergic systems(138). Kopin et al(142) demonstrated the same, in adrenergic nerve terminals in the isolated cat spleen. In this study, perfusion with radioactive ^{14}C-tyrosine was utilised to identify newly synthesised noradrenaline(^{14}C-tyrosine being converted within the nerve terminal to ^{14}C-noradrenaline). Stimulation of the splenic nerves at a high frequency (30 Hz) resulted in release of ^{14}C-noradrenaline having a greater specific activity than that found in the spleen indicating that the newly synthesised noradrenaline is selectively released. When spleens from cats which had been treated with ^{3}H-noradrenaline (instead of ^{14}C-tyrosine) were perfused, the specific activity of ^{3}H-noradrenaline initially was similar to that in the spleen. With continuous stimulation the specific activity fell to about one-third that found in spleen. If the perfusing solution contained α-methyl tyrosine (an inhibitor of noradrenaline synthesis), the initial release was not altered. At later times however, only about one-third as much noradrenaline was released and the specific activity of the released ^{3}-H-noradrenaline was similar to that in the spleen. These results suggest that, during continuous rapid stimulation, it is the new synthesis of the transmitter which plays the key role in maintaining a stable output of noradrenaline. It should be noted that, experiments comparing the specific activity of released compounds with that in the organ face a number of difficulties which may lead to erroneous findings(138). For instance, the homogenisation procedure (utilised in measuring the specific activity in the tissue) used may lead to the release of the transmitter. Mechanical shearing that nerve endings are exposed to during tissue disruption and homogenization will (in the presence of Ca^{2+} ions) cause the release of transmitter from a large number of vesicles.

A study by Hughes and Roth(143) in the rabbit portal vein and vasa deferentia yielded a different result from the study of Kopin et al described above; the tissue stores of transmitter were pre-labelled using a combination of ^{3}H-noradrenaline and ^{14}C-tyrosine. The output per pulse of newly synthesised ^{14}C-noradrenaline remained constant as the train length of TNS was increased while the output per pulse of ^{3}H-noradrenaline increased under the same conditions. This phenomenon was independent of the stimulus frequency. Thus, in this study, the newly synthesised noradrenaline did not appear to be preferentially released during stimulation. Although this study, too, is not without criticism, it is apparent that different sub-cellular compartments of noradrenaline exists in adrenergic nerves. Further, these compartments are probably mobilised in a differential fashion during stimulation of the sympathetic nerves.

A model summarising the characteristics of catecholamine synthesis, catecholamine pools and permeability was put forward by Klein and Lagercrantz(132). According to the model the large dense-core vesicle is believed to contain two noradrenaline pools; a readily saturable fast-release pool for newly synthesised noradrenaline, which exclusively accumulates in this pool and a slower release, ATP-facilitated uptake pool. The fast release pool may be localized at the vesicular membrane together with the enzyme dopamine-β-hydroxylase which is localised at the inner surface of the vesicular membrane. Dopamine is taken up into this pool by a high affinity mechanism which is relatively unaffected by noradrenaline and does not involve ATP. On the other hand, the slow release pool takes up noradrenaline and dopamine by a Mg^{2+}-ATP facilitated uptake system where noradrenaline competes very effectively with dopamine. The neuronal membrane too contains a carrier-mediated uptake for catecholamines (Uptake$_1$). This uptake system and the large dense-core vesicular membrane ATP-facilitated uptake mechanisms show similar, high affinities (K_m approximately 1.0-1.5 μmol) for noradrenaline. The small dense-core vesicles too have an Mg^{2+}-ATP facilitated uptake system but the Km value is much higher (approximately 22 μmol). However, the Km of the intraneuronal metabolising enzyme monoamine oxidase for noradrenaline is approximately 100 μmol. Thus, both vesicle types especially the large vesicles compete very favourably for free noradrenaline in the neuroplasms compared to potential inactivation by monoamine oxidase. Further, a considerably higher level of free noradrenaline in the neuroplasm is also probably needed to inhibit the induction of tyrosine hydroxylase (end-product feed-back inhibition, see above), the rate-limiting enzyme in the synthesis of catecholamines (Km for tyrosine >10 μmol). Thus the events during the synthesis and storage of noradrenaline in the adrenergic nerve endings can be summarised as follows. With the activation of noradrenaline synthesis by dopamine-β-hydroxylase in the large dense-core vesicles, there ensues a rapid, ATP-enhanced overflow of newly synthesised noradrenaline from the fast release pool into the neuroplasm. The noradrenaline in the neuroplasm is taken up by the ATP-facilitated uptake system into the dense-core vesicles, which may have been depleted and are ready to be re-used or which may be locally formed from specialised endoplasmic reticular elements in the nerve terminals. With the relative saturation of the above uptake system with noradrenaline, neuroplasmic levels of noradrenaline would rise and "turn-off" the tyrosine hydroxylase activity.

Although the above mentioned model is both a plausible and an attractive one, more investigations are needed before it can be accepted in total.

The noradrenaline released from the varicosities enters into the synaptic cleft between varicosities and the smooth muscle. The synaptic cleft width can range from 20 nm in the guinea pig vas deferens to 1900 nm in the rabbit pulmonary artery. In general, in the circulation, the smaller the blood vessel, the narrower the cleft, although there are exceptions(144) with the cleft in the canine saphenous vein being about 100-300 nm. Although neuroeffector separation is quite small (15-25 nm) in most non-vascular synapses, there are exceptions; thus the closest neuro-muscular distance in the longitudinal muscle layer of the intestine is about 100 nm(55). The neurogenic response of smooth muscle can be related to the width of the synaptic cleft and the density and distribution of the nerve varicosities. Blood vessels containing a dense innervation have a greater maximum response (in relation to the maximum response to exogenous noradrenaline) during maximum nerve activity and a steeper slope in the frequency response relation. For instance, in the rabbit proximal saphenous artery and in the rat portal vein with dense adrenergic innervations, the maximum response to TNS was 85-90 per cent of the maximum contraction produced by exogenous noradrenaline. In contrast, in the rabbit pulmonary artery which has it nerves confined to the adventitio-medial junction with wide clefts, the maximum response to TNS was approximately 50 per cent of that to exogenous noradrenaline(145). On the other hand, a negative correlation is found between the innervation density and the ED_{50} to exogenous noradrenaline. Bevan determined the concentrations of noradrenaline inside and outside the cleft during the transmural nerve stimulation in terms of steady-state exogenous noradrenaline concentration. In the rabbit pulmonary artery with a wide cleft the two estimates were similar with 1.5 x 10^{-7} and 5.5 x 10^{-7} mol/l inside and outside the cleft respectively. With a narrow cleft as in the rat portal vein the intrasynaptic concentrations were estimated as high as 1.5 x 10^{-5}mol/l while the outside was estimated at 6.0 x 10^{-9} mol/l during stimulation of the adrenergic nerves(146). Thus, a high transmitter gradient may exist between the inside and the outside of the neural cleft when the synaptic distance is small. This is probably due to a mechanical barrier to transmitter egress with a narrow cleft. These studies also suggest that the noradrenaline must be released only from the area of the varicosity facing the smooth muscle, rather than from any part of the surface of the varicosity as otherwise such a big gradient is unlikely to be produced. It is this area only which is usually devoid of a surrounding Schwann cell sheath. It has been observed that the neuronal uptake of tritiated noradrenaline (per unit noradrenaline content) diminishes by as much at 75-80 per cent with diminishing synaptic cleft width(144).

Transmitter disposition and termination of its effect

Diffusion, neuronal uptake, extraneuronal uptake, tissue binding and catabolism constitute the important routes for disposition of released noradrenaline at adrenergic nerve endings. The relative importance of the different pathways varies depending on the density and pattern of innervation, and the width of the synaptic cleft.

In blood vessels with a wide synaptic cleft the immediate movement of the released transmitter into the perisynaptic area is governed by diffusion. However, in a narrow cleft, the movement is restricted within the cleft, and diffusion outward can only occur, from the junction of the synaptic slit with the 'general' extracellular space.

Once the transmitter reaches the perisynaptic area it diffuses in all directions, although initially there is some tangential and longitudinal movement of the transmitter within the nerve plexus due to the scattered sites of release. The transmitter reaches either the tunica adventitia or the tunica media by diffusion, and its distribution between these two layers depends on their relative thickness and on their resistance to transmitter diffusion. The escape of tritiated noradrenaline from the adventitial and intimal sides of the blood vessel during TNS has been investigated by Bevan and Su(146) in the rabbit thoracic aorta and rabbit ear artery. An isolated segment of vessel was perfused (to estimate the overflow of transmitter from the intimal surface) as well as superfused (to estimate the overflow from the adventitial surface) with buffer solution, during transmural nerve stimulation - after labelling the noradrenaline stores with ^{3}H-noradrenaline. In the steady state, in the presence of phenoxybenzamine, the ratio of intimal to adventitial outflow was 0.1 for the pulmonary artery and 0.16 for the ear artery. Presumably in vivo, just as in vitro, there are large sinks at the adventitial and intimal surfaces: the adventitial overflow of noradrenaline being carried away via the adventitial capillary plexus, and intimal overflow entering directly into the circulation. This transmitter entering the circulation constitutes the most important fraction of circulating noradrenaline (most of the circulating adrenaline originates from the adrenal glands). In isolated blood vessels studied under controlled conditions, there is a linear correlation between the changes in overflow of endogenous noradrenaline and changes in the effector response during activation of the adrenergic nerve endings(15,147). Diffusion of transmitter out of the blood vessel wall has not been adequately investigated in most studies of noradrenaline disposition. This is partly because of the difficulties encountered in obtaining proper estimates for diffusion compared with the other disposition mechanisms. It should be noted, however, that the rate of diffusion may exceed that of all other mechanisms.

Uptake$_1$(neuronal uptake)

Neuronal uptake or uptake$_1$ is the active transport of noradrenaline and adrenaline into the axoplasm of the adrenergic nerve endings. This is believed to be one of the main pathways responsible for termination of the action of released noradrenaline at the neural cleft. The characteristics of this membrane carrier system has been reviewed by Iverson(148,149) and are summarised below.

1. Uptake$_1$ process appears to have identical properties in the noradrenaline containing neurones of the peripheral and central nervous systems and results from the activity of a membrane carrier system requiring metabolic energy (Note: The dopamine containing neurones in the mammalian central nervous system have a membrane carrier system analagous to uptake$_1$. However, this uptake system has a very high affinity for dopamine and a lower affinity for noradrenaline).
2. The uptake process is saturable and has a very high affinity for noradrenaline, the transport constant or apparent "Km" being between 0.2 μmol and 1 μmol in most rat tissues.
3. It is stereochemically selective in rat tissues having five times the affinity for the naturally occuring (-)noradrenaline compared with the (+)enantiomer. However, such stereoselectivity seems to be lacking in guinea pig and rabbit tissues.

4. The necessary structural requirements for uptake_1 are: (a) the absence of bulky N-substituent groups (isoprenaline is not a substrate) (b) absence of methoxyl groups on the aromatic ring (normetanephrine and methoxamine are not substrates for uptake_1) (c) presence of at least one phenolic hydroxyl group (amphetamine, phenylethylamine, phenylethanolamine and norephedrine are not substrates).

Depending on the above, various substances are taken up by the adrenergic nerve endings with varying degrees of affinity, e.g. metaraminol, α-methyl noradrenaline, tyramine, octopamine. Adrenaline is also taken up by uptake_1 and has an affinity of about half of that of noradrenaline.

5. As with most other membrane transport systems for organic compounds, uptake_1 is temperature sensitive (approximately doubling of the rate of uptake for an increase in temperature of 10°C) and can be inhibited by metabolic poisons such as dinitrophenol and cyanide or by anoxia.

6. It is dependent on extracellular Na^+ with a marked reduction in uptake when external Na^+ ions are removed. It also requires the presence of a low concentration of K^+ ions (approximately 5 mmol/l) but is inhibited by high concentrations of K^+ (>50 mmol/l). Uptake_1 is also inhibited by inhibitors of membrane Na^+/K^+ ATPase such as ouabain.

7. The structure-activity relationships for inhibition of uptake_1 can be summarised as follows:

(a) affinity for uptake_1 sites is decreased by the presence of bulky substituent groups on the terminal nitrogen of phenylethylamine side chain, by the presence of methoyl substituents on the ring, and by the presence of a hydroxyl group on the β-carbon of the side chain. For the latter compounds, affinity for uptake$_1$ sites is greatest for the isomer corresponding to (-)noradrenaline

(b) affinity is increased by the presence of phenolic hydroxyl groups, particularly in para- and meta- positions, and also by methylation of the α-carbon of the side chain. In the latter case, affinity is highest for the isomer correspondidng to (+) amphetamine.

It should be noted that the structure activity relationships for inhibition of uptake$_1$ by sympathomimetic amines are not identical with the requirements mentioned above, for substrates for the uptake process. For instance, amphetamine or β-phenylethylamine, which lack phenolic hydroxyl groups, do not appear to be substrates for uptake$_1$, but are nevertheless competitive inhibitors of ^{3}H-noradrenaline uptake. This suggests that such compounds, like competitive enzyme inhibitors, are able to bind with high affinity to uptake$_1$ sites in the axonal membrane of adrenergic nerves, but lack the further structural features needed for the inward transport stages which follow the binding. Alternatively, many sympathomimetic amines may compete with noradrenaline for binding to the uptake$_1$ sites and this may be followed by transport of the competing amine into the axoplasm. Thus, indirectly acting sympathomimetic amines not only release noradrenaline from adrenergic nerve-endings, but they also potentiate the actions of the released catecholamine by inhibiting its re-uptake, e.g. tyramine.

8. Many other compounds inhibit uptake_1 apart from the close structural analogues given above. Tricyclic antidepressants such as imipramine, amitryptaline and desmethylimipramine (desipramine) are potent inhibitors with the latter compound being one of the most powerful inhibitors available at present (50 per cent inhibition of ^{3}H-noradrenaline uptake in the rat heart at a concentration of 10^{-8} mol/l). Other inhibitors include the local anaesthetic drug cocaine, adrenergic receptor blocking drugs phenoxybenzamine and chlorpromazine, the monoamine oxidase inhibitors tranylcypromine and phenelzine and the adrenergic neurone blocking drugs bretylium and guanethidine. Uptake_1 is not inhibited by reserpine.

9. There appears to be a temporal dissociation between the activation of the nerve terminal and neuronal uptake process with the latter operating only when the neuronal membrane is polarized(15,150). In the guinea pig vas deferens the uptake of ^{3}H-noradrenaline was shown to be inhibited by stimulation of the effector nerves in a frequency dependent manner(151).

The transmitter taken up by the uptake_1 process is either stored in the vesicles or deaminated by the neuroplasmic enzyme monoamine oxidase. The noradrenaline in the neuroplasm is transported into the storage vesicles by an energy dependent uptake process which is quite separate from the uptake_1 described above(148,152). This uptake is dependent on the presence of ATP and Mg^{2+} and is accompanied by a splitting of the terminal phosphate groups of ATP. This vesicular uptake system is potently inhibited by reserpine, tetrabenazine and prenylamine which are relatively ineffective as $uptake_1$ inhibitors. Reserpine is particularly potent being effective in vitro at a concentration of 10^{-8} mol/l. The vesicular uptake system too, appears to be temperature dependent, energy requiring and stereochemically selective to (-)noradrenaline. The ATP-dependent uptake systems of both the large and small dense-core vesicles have lower transport constants 'Km': 1.5 μmol and 10 μmol respectively) for noradrenaline than the Km of monoamine oxidase for noradrenaline (100 μmol). Thus, most of the noradrenaline taken up into the nerve terminal by $uptake_1$ presumably goes back into the vesicles rather than being enzymatically degraded in adrenergic varicosities.

Following transport of the noradrenaline into the vesicles it forms a tetracatecholamine-ATP complex (molar ratio 4:1) with ATP. This in turn may be bound to the soluble protein chromogranin(152). This vesicular storage function appears to be distinct from the vesicular uptake function even though both functions are inhibited by reserpine(152). The action of reserpine on the vesicular uptake mechanism seems to be competitive with catecholamines, as the presence of high catecholamine concentrations in the vicinity of the reserpine receptor can compete with the drug. On the other hand, once reserpine has interacted with the storage systems, the latter remains inhibited even in the presence of noradrenaline. Thus, the initial action (on vesicular uptake) of reserpine is reversible but the later action (on vesicular storage) irreversible. The recovery is usually accompanied by the arrival in the adrenergic nerve terminal, of new vesicles (from the neuronal cell body) not affected by the drug(152). However, it is not clear as to how the arrival of a relatively few new vesicles could, by themselves, allow the almost complete restoration of the uptake function. The storage function in the vesicles exhibits a greater structural specificity as compared with the vesicular uptake of amine which is relatively non-specific. Structural requirements for storage in adrenergic vesicles are phenylethylamines with a β-hydroxyl group and at least one phenolic group. The molecule also must be of the correct optical configuration corresponding to that of α-noradrenaline(152). It appears that the storage function, as opposed to the uptake function of the vesicle or the uptake$_1$ process (at the nerve membrane) is the absolute determinant of the nature of the amine stored, such that noradrenaline, dopamine and 5-hydroxytryptamine are stored exclusively in their respective neurones despite the close similarities in their neuronal transport systems.

It is generally accepted that uptake$_1$ (neuronal uptake) represents an important mechanism for the termination of action of neurogenic noradrenaline, i.e., noradrenaline released at adrenergic nerve endings. The percentage of neurogenic noradrenaline taken up by the nerve ending varies with the width of the cleft and possibly with the species(73). Inhibitors of uptake$_1$ may have one or more of the following effects on the response of blood vessels to sympathetic nerve stimulation(15): 1) no or only moderate augmentation of the efflux of noradrenaline; with cocaine this effect may be partially obscured by the local anaesthetic properties of the compound (which would tend to decrease the release of ^{3}H-noradrenaline). 2) the appearance of deaminated metabolites especially 3,4-dihydroxyphenylglycol(DOPEG) is reduced; DOPEG is formed by the action of monoamine oxidase (MAO) on the noradrenaline, subsequent to uptake$_1$. 3) a moderate augmentation of the contractile response to low-frequency stimulation occurs - there is usually no augmentation with higher frequencies; this minimal effect is probably due to the fact that uptake$_1$ is inhibited during sympathetic nerve stimulation in a frequency dependent manner(151). Thus, an uptake$_1$ inhibitor may not change the transmitter concentration at the synaptic cleft during nerve stimulation to any appreciable degree. 4) the contractile response, however, tends to become prolonged and the relaxation delayed; this occurs as uptake$_1$ plays an important role in removing the transmitter from the receptor site once the stimulation is terminated. Blockade of this would lead to a prolonged effect while not significantly affecting the magnitude of the concentration of the noradrenaline (and thus the magnitude of the contraction) at the cleft.

Uptake$_1$ inhibitors also potentiate the responses to exogenous catecholamines. The magnitude of this potentiation too depends on the density of innervation and the width of the synaptic cleft and also on the particular catecholamine studied(149). Thus uptake$_1$ inhibitors potentiate the effects of noradrenaline to a greater extent than those of adrenaline which has a lesser affinity for the uptake process. Potentiation of the effects of sympathomimetic amines will only occur if the following conditions are fulfilled(149): 1) the agonist is a substrate for uptake$_1$ with an affinity constant = K. 2) the agonist elicits a pharmacological response in a dose range at which uptake$_1$ remains unsaturated, that is $ED_{50} < K$. In general, potentiation will thus occur only in the case of potent agonists. The effect is greatest in tissues such as the cat nictitating membrane with a dense adrenergic innervation, and not at all in tissues lacking sympathetic innervation. Also in tissues in which the neuromuscular interval is small the potentiation by uptake_1 inhibitors will be greater(153); the uptake_1 process in general would significantly alter the cleft concentration of noradrenaline only in the case of a narrow cleft in spite of the fact that the rate of diffusion of exogenous noradrenaline into a narrow cleft would actually be less than that into a wide cleft (see above).

In addition to the above, the route of administration of the catecholamine may play an important role in the magnitude of the potentiation produced by uptake_1 inhibition in isolated blood vessels. De La Lande et al.(154) in a study on the isolated, perfused, central ear artery of the rabbit, demonstrated that while cocaine greatly potentiated the effects of extraluminally administered noradrenaline, it had little effect on the sensitivity to intraluminally administered noradrenaline. Surgical interruption of the sympathetic supply to the artery 14-24 days prior to the experiment simulated the effects of cocaine described above. Cocaine itself had no significant effect following the surgical denervation. These observations are probably explained by the fact that the extraluminally applied noradrenaline has to diffuse through the adventitia into the media, before it can exert its effect on the smooth muscle; as the dense adrenergic nerve plexus supplying the media of this vessel is located in the inner adventitia some of the extraluminally administered noradrenaline would be removed by uptake_1 - prior to exerting its action. This would result in a reduction in the concentration of noradrenaline achieved in the tunica media thus limiting the response to the drug. Cocaine and denervation, by blocking the uptake process, leads to a supersensitivity to extraluminally administered noradrenaline only. However, studies using cocaine are complicated by the fact that the drug has a post-junctional sensitising action in addition to the uptake$_1$ blockade. Thus, it potentiates the responses to the α-agonist methoxamine (which is not a substrate for uptake$_1$) in the rabbit aorta(153). Osswald in a similar study in the canine lateral saphenous vein(17), demonstrated that cocaine potentiated ~~the~~ effects of both intraluminally and extraluminally applied noradrenaline to the same extent. This is probably due to the fact that in the canine saphenous vein adrenergic nerves are distributed throughout the tunica media.

Uptake$_2$ (Extraneuronal uptake)

This is a transport system for catecholamines, present in smooth muscle, cardiac muscle, endothelium and certain glandular tissues(148,155). This system has a much lower affinity for noradrenaline and adrenaline than uptake$_1$ (Km for (-)noradrenaline 0.27 μmol and 252 μmol for uptake$_1$ and uptake$_2$ respectively in rat heart tissue). However, uptake$_2$ has a very much higher capacity than uptake$_1$ although it is saturable. As the accumulated catecholamine is not firmly retained but rapidly metabolised by MAO and catechol-o-methyltransferase (COMT), uptake$_2$ is not usually detectable at low concentrations of catecholamines. Uptake$_2$ does not demonstrate a stereochemical specificity for (+) or (-)noradrenaline or adrenaline and has a higher affinity for the latter. It has quite a different substrate specificity from uptake$_1$; isoprenaline, an amine not taken up by uptake$_1$, is a better substrate than even adrenaline for uptake$_2$. The structure-activity relationship for the inhibition of uptake$_2$ by sympathomimetic amines are almost the converse of those found for uptake$_1$(148); thus inhibition of uptake$_2$ was enhanced by N-substitution and by O-methylation, normetanephrine and metanephrine being potent inhibitors. On the other hand (-)metaraminol, a potent inhibitor of uptake$_1$ had no inhibitory effect on uptake$_2$. Phenoxybenzamine too is a potent inhibitor of uptake$_2$ although its usefulness as a tool to block uptake$_2$ is limited due to its other actions such as blockade of uptake$_1$ and the blockade of α-receptors. Steroid compounds have also been discovered as uptake$_2$ inhibitors, β-oestradiol and corticosterone being quite potent. Uptake$_2$ is less dependent on Na^+ ions compared to uptake$_1$. The effects of anoxia, cooling and ATP deprivation are also not as clear as with uptake$_1$(155).

Unlike uptake$_1$, the role played by uptake$_2$ in the termination of the actions of catecholamines released at sympathetic nerve endings is likely to be minor. However, under conditions in which uptake$_1$ is blocked (eg. by administration of tricyclic anti-depressants) uptake$_2$ may assume a bigger role. On the other hand, because of the widespread distribution of uptake$_2$ sites in vascular smooth muscle, and the preference for adrenaline as substrate, it is likely that uptake$_2$ plays an important role in the rapid removal and inactivation of circulating catecholamines(148).

Some isolated smooth muscle such as the rabbit aorta and the rabbit ear artery show a potentiation of the effects of catecholamines with blockade of uptake$_2$(153), although the potentiation is much less compared with that observed following blockade of uptake$_1$. In the perfused rabbit ear artery, responses to both intraluminal and extraluminal noradrenaline are potentiated and the supersensitivity is not decreased by denervation or cocaine. Another important feature of the uptake$_2$ process is that in contrast to the adrenergic nerve ending, extraneuronal uptake has little or no capacity for storage of unchanged amines (Note: capacity for uptake is however, high). The extent to which uptake$_2$ influences the concentration of catecholamines at the receptors is ultimately dependent on metabolism by COMT. Thus if COMT is inhibited, blockade of extraneuronal uptake does not lead to supersensitivity. These findings suggest that uptake$_2$ and metabolism by COMT, are arranged in series so that blockade of one process eliminates the effects of blocking the second. Thus, for supersensitivity to occur the agonist has to fulfill two criteria: (1) it must be a substrate for uptake$_2$. (2) it must be a substrate for the enzyme COMT(153). For instance, phenylephrine, although a substrate of uptake$_2$, is not metabolised by COMT as it is not a catechol compound. Thus, the response of the nictitating membrane to phenylephrine is not potentiated by hydrocortisone, an uptake$_2$ inhibitor.

Inhibition of uptake$_2$ may have one or more of the following effects on the response of blood vessels to sympathetic stimulation(15): 1) no, or modest increase in the overflow of ^{3}H-noradrenaline. 2) reduced appearance of extraneuronal metabolites of noradrenaline (eg. normetanephrines). 3) augmentation of the contractile response. 4) moderate prolongation of the contractile response and delayed relaxation.

Catabolism(156,157)

Chemical inactivation of noradrenaline in adrenergic neuroeffector systems is controlled by two enzymes, monoamine oxidase(MAO) and catechol-o-methyltransferase(COMT). The enzyme MAO which catalyses the deamination of a wide variety of amines is widely distributed in the body with the liver, kidney, intestines, stomach and aorta constituting rich sources(156). It is either absent, or present in very low amounts in skeletal muscle, plasma and erythocytes, although it occurs in platelets. It has also been demonstrated in a variety of blood vessels(15). Despite this wide distribution, monoamine oxidases isolated from different organs in species show considerable differences in specificity. The enzyme is located almost exclusively in the outer membrane of the mitochondria, inside cells. The minimum molecular weight of the enzyme has been estimated at 100,000 although it can vary up to one million depending on the preparation(156). The enzyme exists in two main forms, MAO-A and MAO-B. Adrenergic nerves contain mainly type A and the vascular smooth muscle cells, probably type B. The total MAO content of the rabbit ear artery is reduced only 10 per cent by chronic sympathetic denervation indicating the larger size of the extraneuronal stores of the enzyme. However, this relatively small amount of neuronally localised MAO, metabolises the transmitter released at the nerve ending and is thus functionally important in the adrenergic neuro-effector interaction(73). Extraneuronal MAO is effective only against high concentrations of noradrenaline, and its inhibition has minimal influence on the responses to endogenous or exogenous noradrenaline(73, 158).

The enzyme COMT, like MAO, is also widely distributed in the body including the brain, with high concentration in the liver and kidney(130). However, unlike MAO, it is located in the cytoplasm and has no selective association with adrenergic nerve endings. Most of the

COMT activity of the blood vessels is extraneuronally located, presumably in the vascular smooth muscle cells. Catabolism of catecholamines in the body results from a combination of actions from the two enzymes at neuronal and extraneuronal sites. Oxidative deamination of noradrenaline by MAO results in the formation of 3,4-dihydroxyphenyl glycoaldehyde (DOPAL). The aldehyde metabolites of catecholamines can be demonstrated in vitro, but are rarely detected in tissues or urine. This is because these aldehydes are immediately metabolised to more stable products. The oxidation of DOPAL by aldehyde dehydrogenase leads to the formation of 3,4-dihydroxymandelic acid (DOMA). The reduction of DOPAL by aldehyde reductase (alcohol dehydrogenase) results in the formation of 3,4-dihydroxyphenylglycol (DOPEG). On the other hand, action of COMT on noradrenaline results in methylation at the ortho-position with the formation of normetanephrine (NMN). The NMN may be acted upon by MAO to form an unstable aldehyde which in turn can be oxidised or reduced by aldehyde dehydrogenase and aldehyde reductase respectively. This results in the formation of 3-methoxy-4-hydroxymandelic acid (incorrectly called vanillylmandelic acid-VMA) and 3-methoxy-4-hydroxyphenylglycol (MOPEG) respectively. 3-methoxy-4-hydroxymandelic acid ("VMA") constitutes the major metabolite of catecholamines excreted in the urine. The corresponding product of the metabolic degradation of dopamine, which cantains no hydroxyl group in the side chain, is homovanillic acid (HVA).

A significant portion of noradrenaline spontaneously leaking out of the storage vesicles in the resting state and that displaced by indirectly acting sympathomimetic agents are acted upon by MAO, with the ultimate production of DOPEG as the major metabolite under those circumstances. Some of the noradrenaline that is taken up (by $uptake_1$) following exocytotic release too is subjected to oxidative deamination by MAO with the production of DOPEG and DOMA as the end products. These two compounds may possibly be o-methylated (by COMT) in extraneuronal tissues with the formation of MOPEG and VMA. Some of the noradrenaline released during sympathetic nerve stimulation is taken up by $uptake_2$ into smooth muscle cells as explained before. This noradrenaline is degraded by COMT which is predominantly located extraneuronally with the formation of NMN. As extraneuronal uptake plays a major role in the disposition of circulating catecholamines, relatively more NMN and the other two o-methylated prducts (MOPEG and VMA) are formed under these circumstances. The relative proportions of the different metabolites formed seem to differ not only with the mode of adrenergic stimulation (exocytotic release, displacement release, stimulation by circulating catecholamines) but also with tissue and species variation(157,159).

Inhibitors of monoamine oxidase may have one or more of the following effects on the response to sympathetic nerve stimulation(15): 1) decreased appearance of deaminated and o-methylated deaminated metabolites, and moderate augmentation of the overflow of intact transmitter. 2) moderate (or no) augmentation of the contractile response. 3) moderate (or no) prolongation of the contractile response and delayed relaxation. In addition they may augment and prolong the response to indirect sympathomimetic amines and to exogenous noradrenaline. Analysis of the effects of these inhibitors are made difficult by the fact that these drugs by themselves can cause pharmacological displacement of the stored transmitter and facilitate the release evoked by adrenergic stimulation. Supersensitivity to exogenous noradrenaline produced by MAO inhibitors usually occurs only in innervated muscle. Moreover, in the perfused rabbit ear artery preparation, nialamide (a MAO-inhibitor) potentiates the response to extraluminally administered noradrenaline but not to intraluminally applied noradrenaline. These findings suggest that the supersensitivity produced by MAO inhibitors is probably due to inhibition of the neuronally localised MAO, rather than the extraneuronally localised enzyme(153). Thus, the supersensitivity following inhibition of MAO is more pronounced in those tissues in which uptake$_1$ plays a major role in the disposition of the transmitter.

Inhibition of COMT by drugs such as pyrogallol, tropolone and U-0521 may have the following effects on the response to sympathetic nerve stimulation in smooth muscle(15): 1) decreased appearance of o-methylated and o-methylated deaminated metabolites. 2) augmented outflow of intact transmitter and deaminated metabolites. 3) moderate (or no) augmentation of the contractile response. 4) moderate (or no) prolongation of the contractile response. They may also augment and prolong the contractile response of smooth muscle to exogenous catecholamines. This supersensitivity is probably due to the build-up of noradrenaline in the muscle cells with resultant slowing down of the extraneuronal uptake process, as the two processes seem to be coupled in series as explained before. Conversely, if extraneuronal uptake is blocked by steroids, then inhibition of COMT does not produce supersensitivity. In the isolated perfused rabbit ear artery, inhibition of COMT potentiates the responses to both intraluminally and extraluminally applied noradrenaline to the same extent. In addition, the effects of COMT inhibitors are not affected by prior treatment with

$uptake_1$ blockers. These findings indicate that the supersensitivity is probably due to the inhibtion of extraneuronal COMT rather than the intraneuronal enzyme (c.f. with MAO inhibitors)(153). Supersensitivity following COMT inhibition is related to the sensitivity of the tissue to the catecholamine rather than to the functional state of the $uptake_1$ mechanism. This occurs because the o-methylating system is easily saturated. Thus, supersensitivity occurs only in tissues in which the sensitivity to catecholamines is high.

Binding of catecholamines to connective tissue

A part of the noradrenaline released at nerve endings is believed to bind to connective tissue. The role of catecholamine binding to collagen and elastin was investigated by Powis(160). Collagen showed no specificity towards binding of either (-) or (+)adrenaline and noradrenaline while elastin bound the (-)isomers to a greater extent. Both elastin and collagen demonstrated two sites of binding: a high affinity, limited capacity site and a low affinity, high capacity site. Tetracyclines inhibited this binding, with oxytetracycline in a concentration of 10^{-4} mol/l inhibiting the binding of noradrenaline to collagen by 68.4 per cent. Oxytetracyline (10^{-4} mol/l) was shown to potentiate the amplitude of the response of the rabbit ear artery to noradrenaline and to nerve stimulation ten- and six-fold respectively. As blood vessels contain a relatively high proportion of elastin and collagen (over 60 per cent of the dry weight in some vessels) connective tissue binding of catecholamines could theoretically exert a considerable effect on the magnitude of the responses to these compounds. In the rabbit ear artery the potentiation produced by blockade of $uptake_1$ and $uptake_2$ were much less than the potentiation produced by oxytetracycline. However, some studies did not show any potentiation of the responses to neural stimulation and exogenous catecholamines by tetracyclines(161). Thus the importance of the connective tissue binding of catecholamines (as with the other mechanisms of disposition) may vary depending on the tissue as well as with the relative role played by each of the other mechanisms in a particular tissue.

Relative importance of the disposition pathways

Although the existence of several disposition pathways of noradrenaline in vascular tissue is well established, the relative role played by each in a particular blood vessel remains unresolved in most preparations(73). The relative roles are particularly affected by 1) the density of the adrenergic innervation, 2) the anatomical arrangement of the nerve endings within the blood vessel, 3) the width of the junctional cleft, 4) the content of collagen and elastin in a particular blood vessel and 5) whether neurogenic noradrenaline or circulating noradrenaline is being investigated(15). If the cleft is narrow, uptake_1 usually plays a prominent role; if it is wide, diffusion and extraneuronal uptake assume a greater role. The investigations designed to answer this question in a particular blood vessel are complicated by the fact that interactions between these mechanisms are frequent. Therefore, controlling one mechanism by itself may alter the magnitude of the role played by another mechanism, thus confounding the results.

Transmitter disposition in the canine saphenous vein

The relative role of the disposition pathways in the canine lateral saphenous veins were investigated by Guimaraes, Osswald and co-workers(17,162,163). In these studies the uptake_1 inhibition by cocaine

(10^{-5} mol/l) shifted the dose-response curve to exogenous noradrenaline and the stimulus-response curve to TNS, to the left by a factor of 7.1 and 4.5 respectively(162). The difference in the extent of potentiation between exogenous noradrenaline and TNS, which was significant, may have been due to the local anaesthetic effects of cocaine leading to a decrease in the release of endogenous noradrenaline during TNS. Thus, cocaine would have two opposing effects during TNS: blockade of uptake$_1$ leading to potentiation of the contractile response and inhibition of release of the transmitter leading to a decrease in the response. This discrepancy between the extent of potentiation of TNS as compared with exogenous noradrenaline could also be explained by the fact that uptake$_1$ is already blocked to some extent during TNS (uptake$_1$ is believed to be not operative when the nerve terminal is depolarized). This would result in a lesser potentiation by the cocaine. The potentiation of exogenous noradrenaline responses in canine saphenous veins was also produced by surgical denervation 8-10 days prior to the experiment. Cocaine had no further effect in these veins following the surgical denervation.

The authors also measured the time required for half-relaxation following TNS by the oil immersion technique(73,162). Cocaine increased this time 2.9 times. The uptake$_2$ inhibitors U-0521 and cortexone had no significant effect on the time of relaxation suggesting that COMT and uptake$_2$ do not represent important pathways for the inactivation of the released transmitter. The MAO-inhibitor iproniazid caused a small increase in the time needed for half-relaxation. After uptake$_1$ had been blocked by cocaine, iproniazid did not produce a further increase in relaxation time. However, both U-0521 and cortexone produced further increases in the relaxation times with prior cocaine treatment. Thus both COMT metabolism and uptake_2 are able to compensate for the loss of uptake_1. This may, in fact, be due to the increase in the sensitivity to endogenous noradrenaline produced by cocaine resulting in an effective concentration range for the noradrenaline, at which COMT and uptake_2 are no longer saturated as explained above. In summary, it appears that neuronal uptake represents the most important pathway of inactivation for endogenous noradrenaline in the saphenous vein.

Osswald et al(17) also investigated the relative roles played by the different disposition pathways, during the relaxation after a contraction induced by exogenous noradrenaline by the oil-immersion technique. Iproniazid pre-treatment increased the time to 50 per cent relaxation 3.6 fold while cocaine and imipramine augmented the time only 2.6 fold. Tropolene (a COMT inhibitor) caused only 1.38 fold increase. When the three drugs (MAO inhibitor, uptake_1 inhibitor and COMT inhibitor) were used in combination (changing the order of addition), iproniazid added after tropolone and cocaine produced the biggest prolongation of the relaxation time. The authors concluded that oxidative deamination by MAO was the most important pathway for disposition of exogenous noradrenaline in the canine saphenous vein. However, when this problem was investigated by studying the extent of potentiation of the contraction to exogenous noradrenaline as the tool of measurement, blockade of uptake_1 was found to produce the greatest potentiation in the canine saphenous vein.

Guimaraes and Paiva(163) investigated the uptake and metabolism of ^{3}H-noradrenaline and ^{3}H-adrenaline in the canine saphenous vein. Of the ^{3}H-adrenaline taken up by the tissue (during a 30 minute incubation) 70 per cent was metabolised. Deaminated o-methylated metabolites (MOPEG and VMA) represented 59 per cent and o-methylated metabolites (metanephrine) respresented 30 per cent of the total metabolised. The deaminated metabolites (DOPEG and DOMA) represented the balance 10 per cent. Cocaine reduced the removal of ^{3}H-adrenaline by the tissue to 80 per cent of the control value. This reduction was mostly of the unmetabolised (stored) fraction which would presumably be located in the adrenergic nerve terminals and thus blocked by cocaine. There were no major changes in the proportions of different metabolites. The uptake$_2$ blocker deoxycorticosterone, on the other hand, increased the unmetabolised fraction to 45 per cent while reducing the proportion metabolised. Further, the pattern of biotransformation was altered with the deaminated, o-methylated metabolites being reduced to 38 per cent and the o-methylated fraction to 6 per cent.

In the case of ^{3}H-noradrenaline taken up by the tissue, 40 per cent was metabolised with 60 per cent remaining as intact noradrenaline. The deaminated metabolites (DOPEG, DOMA) represented 38 per cent and the o-methylated, deaminated metabolites (MOPEG and VMA) 47 per cent of the total metabolised. Uptake$_1$ inhibition reduced the removal of ^{3}H-noradrenaline to 45 percent of the control value. This reduction was due to a marked decrease in the unmetabolized fraction as in the case of ^{3}H-adrenaline. Although the metabolised fraction did not change appreciably, DOPEG formation was reduced to 13 per cent and NMN formation increased to 27 per cent. Deoxycorticosterone, on the other hand, produced a small decrease in the removal of ^{3}H-noradrenaline with almost all the reduction confined to the metabolised fraction. Again, the production of o-methylated metabolites (NMN) was almost totally abolished with a reduction also in the o-methylated, deaminated fraction. In summary, it could be stated that deamination predominated in the metabolism of ^{3}H-noradrenaline and o-methylation in the metabolism of ^{3}H-adrenaline in the saphenous vein. The metabolism of ^{3}H-adrenaline was markedly reduced by extraneuronal uptake blockade. The study also demonstrated that a significant amount of DOPEG formation occurred extraneuronally too when higher concentrations (2.3 μmol/l) of the tritiated compounds were used during the incubation period. Thus, in contrast to many other tissues in which extraneuronal uptake plays a minor role in the disposition of catecholamines, in the dog saphenous vein it plays a significant role in inactivation of exogenous catecholamines especialy adrenaline.

In a more recent study Muldoon et al(159) analysed the metabolic fractions during TNS, following labelling of the tissue stores with ^{3}H-noradrenaline. Under basal conditions intact noradrenaline accounted for only a small fraction of the total spontaneous efflux of tritiated material. The deaminated metabolites (DOPEG and DOMA) represented the main fraction with the amount of DOPEG being 4-8 times that of DOMA. The glycol, MOPEG was the largest component among the o-methylated metabolites (MOPEG, VMA, NMN) and NMN the smallest. During TNS the amount of intact ^{3}H-noradrenaline in the superfusate increased, and it formed the largest fraction at frequencies of 2Hz and higher. Among the metabolites NMN showed the most striking increase followed by MOPEG. Both DOPEG and DOMA also increased with increasing frequency while VMA showed a much smaller increase. During TNS following blockade of uptake$_1$ by cocaine (10^{-5}mol/l), all metabolite fractions increased except DOPEG. Cocaine did not alter the basal efflux appreciably. On the other hand, the MAO-inhibitor pargyline produced a decrease of the total radioactivity in the basal state with a decrease in all deaminated compounds except DOMA. However, the efflux of intact noradrenaline and NMN showed a significant increase. During TNS at 2 Hz after pargyline pre-treatment, there was an increased amount of total radioactivity in the superfusate accompanied by increases in intact ^{3}H-noradrenaline and NMN compared with the control state. The amount of DOPEG was less than in the controls. In this study the amount of DOMA in the superfusate was consistently less than the amount of DOPEG. This suggests that, in the saphenous vein, after deamination of noradrenaline, the formed aldehyde is reduced by aldehyde reductase (to the glycol DOPEG) rather than oxidised by aldehyde dehydrogenase to the acid DOMA. An alternative explanation is that DOMA formation takes place mainly extraneuronally. This would explain why cocaine did not reverse the efflux pattern of DOMA. In the case of the o-methylated metabolites, the glycol MOPEG was always found in excess of VMA in the superfusate. Thus, here too the aldehyde reductase appears to play the more dominant role. The o-methylated, deaminated metabolites could be produced by either the nerve terminals or in the extraneuronal tissue. As cocaine augments the evoked release of o-methylated deaminated compounds during TNS (while depressing efflux of DOPEG), the main site of origin for these metabolites must be outside the neurones, presumably in smooth muscle cells.

It should be noted that in all experiments utilising tritiated catecholamines, the possibility exists that the disposition of the ^{3}H-noradrenaline may not be the same as that of endogenous noradrenaline. The studies discussed above illustrate the differences observed when different techniques are used to investigate the relative role of the disposition pathways. The studies by Guimaraes and Osswald(17) where the effector responses were investigated, suggested a predominant role for uptake$_1$ and deamination by MAO in the disposition of noradrenaline. The other studies(159,163) where metabolite fractions were measured, do not indicate a major role for the above pathways, but stress the role of extraneuronal uptake. This is probably due to the fact that the criterion measured is different in the two sets of studies (effector response and metabolite fractions) and thus do not mean the same in physiological terms.

Pre-synaptic modulation of transmitter release

Three groups of receptors are generally involved in chemical neurotransmission(164). 1) The receptors on the soma and dendrites of the innervating neurone which determine the frequency of impulses carried down to the axon terminals. 2) The post-synaptic receptors which recognise the transmitter and mediates the response of the innervated cells. 3) While these two receptors would be adequate for the system to produce an effector response, there is evidence that a third group is located on the nerve endings modulating the release of the transmitter and in some cases its synthesis. These receptors are known as pre-junctional or pre-synaptic receptors. Biochemical experiments that retrospectively can be explained by pre-synaptic receptors date back to the 1950's(165,166). Since these initial reports a large number of publications have confirmed that α-adrenoceptor blocking agents, like phenoxybenzamine, increase the overflow of norepinephrine elicited by nerve stimulation. These findings were initially attributed to, blocking a site of loss for the transmitter at the α-receptors, the blocking of neuronal uptake and the blocking of extraneuronal uptake by these α-adrenoceptor blocking agents. The first of these possibilities was later found to be untrue and the other two possibilities, although true, not sufficient by themselves to account for the magnitude and the pattern of increase in overflow of noradrenaline and its metabolites observed with α-receptor blocking agents. The evidence against these hypotheses has been reviewed by Langer(5) and Westfall(129). 1) Cocaine and desmethylimipramine (desipramine) which are potent inhibitors of $uptake_1$ produce only a small increase in efflux of ^3H-noradrenaline with nerve stimulation in contrast to phenoxybenzamine which produced a marked increase in efflux. 2) α-adrenoceptor blocking agents produce a further increase in efflux when added, following blockade of $uptake_1$ by a maximal dose of cocaine or desipramine. 3) α-adrenergic blockers increase the efflux of noradrenaline in doses that do not block neuronal uptake. 4) The α-adrenoceptor antagonist phentolamine which does not block extraneuronal uptake also brings about an increased efflux of noradrenaline during nerve stimulation. Thus extraneuronal uptake cannot account for the increased release of the transmitter. 5) Inhibitors of COMT and inhibitors of $uptake_2$ such as normetanephrine produce a small or no increase in the stimulation-induced efflux of noradrenaline. 6) GD 131, a β-haloalkylamine similar to phenoxybenzamine, that inhibits both $uptake_1$ and $uptake_2$ without blocking α-receptors produces only a slight increase in the stimulation induced efflux of noradrenaline(129). 7) Accompanying the increased efflux of noradrenaline produced by α-adrenergic antagonists, an increased overflow of the intravesicular enzyme dopamine-β-hydroxylase is also observed. Since this enzyme is a rather large molecule that is not taken by uptake$_1$ or uptake$_2$, or subjected to inactivation in the tissue after its exocytotic release, an increase in overflow of the enzyme does indeed represent an actual increase in release at the adrenergic nerve terminal.

These findings led to the hypothesis of pre-synaptic regulation of transmitter release at the adrenergic nerve ending put forth independently by four laboratories in 1971(167,168,169,170). The hypothesis proposed the existence of α-adrenergic receptors on the pre-synaptic membrane at the adrenergic nerve endings; these, when stimulated by the released noradrenaline, would lead to a decrease in further release of the transmitter, i.e. a negative-feedback control of release.

Evidence available for the existence of pre-synaptic α-receptors as proposed could be summarised as follows(15,129). 1) A number of α-adrenergic antagonists including phenoxybenzamine, phentolamine, dihydroergocryptine have been shown to increase the overflow of noradrenaline during nerve stimulation in concentrations that have a minimal or no effect on neuronal and extraneuronal uptake. In some cases, but not all, the increase in overflow of noradrenaline is accompanied by an increase in the effector response in the preparation. 2) α-antagonists lead to a facilitation of the release of noradrenaline from tissues (such as the heart) which are believed to contain relatively few post-synaptic α-receptors. This suggests that the receptors involved in the regulation of the transmitter release are located pre-synaptically (and argues against a trans-synaptic regulation where the post-synaptic effects of the transmitter leads to the formation of a mediator substance which subsequently acts pre-synaptically to regulate the transmitter release). 3) Phenoxybenzamine has been shown to increase the stimulation induced efflux of noradrenaline from axonal sprouts of cultured ganglia where there is no evidence of post-synaptic innervation. Thus, the site of action has to be neuronal. Similar experiments with synaptosomes (resealed torn-off axon terminals) too suggests a direct-action. However, it should be noted that synaptosomes often have post-synaptic membranes attached, so a trans-synaptic action cannot be definitely excluded(171). 4) High K^+ (external) solutions lead to release of neurotransmitters by a direct depolarizing action on nerve terminals: tetrodotoxin, the fast Na^+ channel inhibitor blocks the traffic of action potentials. Hence, when a drug (e.g. α-agonist or α-antagonist) modifies transmitter release induced by high K^+ in the presence of tetrodotoxin, it cannot be acting by way of action potentials in interneurones or by an action potential propagated down to the nerve terminal from the nerve cell body. It must act either on the terminals under study or on neighbouring cells. This model has been used to exclude interneuronal pathways in the α-adrenergic inhibition of noradrenaline release(171,172). 5) A wide range of α-agonists including noradrenaline, clonidine and oxymetazoline have been demonstrated to decrease the stimulation-induced efflux of noradrenaline in a variety of tissues unrelated to a post-synaptic effect(129). This effect can be blocked by the simultaneous use of α-adrenoceptor antagonists.

Further investigations on the α-receptor mediated inhibition of transmitter release resulted in the discovery that pre- and post-synaptic α receptors differed from each other in their pharmacological characteristics(129). When the two types of receptors were compared in the same preparation, a marked difference in potency was noted between different α-agonists on the two receptor types. For instance, in strips of rabbit pulmonary artery(173), methoxamine and phenylephrine either did not change or enhanced, but never reduced, the contractile response to transmural nerve stimulation. In contrast oxymetazoline, α-methylnoradrenaline and tramazoline at low concentrations selectively inhibited the response to transmural nerve stimulation. The rank order of potency for the reduction of stimulation induced efflux of ^{3}H-noradrenaline by 20 per cent (EC_{20} pre) was adrenaline>oxymetazoline >tramazoline>α-methylnoradrenaline>naphazoline>phenylephrine >methoxamine. On the post-synaptic side the rank order of potency for producing 20 per cent of maximum contraction (EC_{20} post) was adrenaline>oxymetazoline>naphazoline>phenylephrine>tramazoline>α-methylnoradrenaline>methoxamine. The ratio EC_{20} pre/EC_{20} post was calculated for each agonist as an index of its relative pre- and post-synaptic potency. According to the ratio the agonists were arbitrarily classified into three groups: group 1 (ratio about 30) - preferentially post-synaptic agonists e.g. methoxamine, phenylephrine; group 2 (ratio around 1)-similar pre-and post-synaptic potencies e.g. adrenaline, naphazoline; group 3 (ratio below 0.2)-preferentially pre-synaptic agonists e.g. oxymetazoline, α-methylnoradrenaline and tramazoline. Although this method is not without error it illustrated the differences between the pre- and post-synaptic receptors with regards to potency of different agonists. Similarly, the effectiveness of different α-antagonists at the two receptor sites in a single tissue was found to be different. These differences in pharmacologic profiles suggested that there is a fundamental difference in the structural requirements for binding at the two receptors. This resulted in the classification of the post synaptic receptor as α_1 and the pre-synaptic receptor as α_2 as originally suggested by Langer(5) and later adapted universally following the review by Berthelsen and Pettinger(6). The studies involving the determination of relative pre- and post-synaptic potencies of agonists demonstrated a fairly consistent pattern of the relative order of potency for the pre-synaptic effects of different agonist between different tissue preparations. However, the relative post-synaptic potencies of the different drugs showed discrepant findings in different vessels. In particular, some apparently pre-synaptically selective drugs were found to be quite effective on the post-synaptic side as well. This was not well understood at the time the above experiments were carried out. However, with the discovery of highly selective α_1 and α_2 antagonists it became clear that α_2-receptors were present on the post-synaptic membrane as well. As the relative numbers of α_1 and α_2 receptors on the smooth muscle cells (i.e. post-synaptically) would differ from tissue to tissue the above mentioned discrepancies would no longer be entirely unexpected.

The mechanism by which pre-synaptic α_2 receptor stimulation leads to a decrease in the release of noradrenaline is believed to be mediated by Ca^{2+} ions(129). Exocytotic release of catecholamines is triggered by an increase in neuroplasmic Ca^{2+} ions. Pre-synaptic α_2-receptor stimulation probably leads to a decrease in the availability of the Ca^{2+} ions involved in this neurosecretory coupling. The available evidence can be summarised as follows.

1) The release of noradrenaline from adrenergic nerve endings produced by both transmural nerve stimulation and high K^+

solution is dependent on an influx of Ca^{2+} ions with exocytosis of the storage vesicles. In contrast, the indirectly acting sympathomimetic agents release noradrenaline from nerve endings by displacement without producing an increase in neuroplasmic Ca^{2+} ions. Lorenz et al(174) in a study in the isolated canine saphenous vein demonstrated that while noradrenaline inhibited the release of ^3H-noradrenaline produced by both K^+ and TNS, it had no effect on the release produced by tyramine, an indirect sympathomimetic agent. Starke and Montel(175) found a similar differential effect in the isolated, perfused rabbit heart with both α-agonists as well as α-antagonists.

2. The magnitude of the pre-junctional inhibitory effect produced by α-agonists has been found to be a function of the extracellular Ca^{2+} concentration(129). Thus, lowering the extracellular Ca^{2+} from that normally present in the superfusion fluid (2.5) to 1.0 mmol/l resulted in a dramatic potentiation of the inhibitory effect of clonidine on stimulation induced noradrenaline efflux(176). Conversely, raising the Ca^{2+} concentration to 5.0 mmol/l prevented the inhibitory effects of clonidine. A similar Ca^{2+} dependency has been observed in the pre-junctional inhibition of adrenergic neurotransmission produced by adenosine in the canine saphenous vein(177).

3. The pre-junctional inhibition of noradrenaline release by activation of α-receptors has a negative correlation with the frequency of stimulation of adrenergic nerves(129,178,179). This too is consistent with a mediation via Ca^{2+} ions.

Presumably, at higher frequencies of stimulation, more Ca^{2+} is available in the neuroplasm overriding the inhibition produced by the activation of pre-synaptic α-receptors.

Although the evidence in favour of Ca^{2+} as the mediator during pre-synaptic α_2 inhibition is convincing, how the α_2-receptor activation limits the availability of Ca^{2+} is not known at present. Theoretically, there could be a decreased influx or an increased efflux of Ca^{2+} or some alteration in Ca^{2+} utilisation (e.g. by affecting its affinity or binding to sites important for exocytosis) at the adrenergic nerve terminal.

Pre-junctional inhibition may partly account for the observation that $uptake_1$ blockers such as cocaine or desipramine produce only a mild to moderate increase in ^3H-noradrenaline overflow during TNS although $uptake_1$ is believed to play an important role in the disposition of neurally released transmitter. This may be due to the higher cleft concentration of noradrenaline produced by the $uptake_1$ blockade inhibiting its own release during TNS. Thus, the actual release of noradrenaline may in fact be less during blockade of neuronal uptake. The other factors that might contribute to this apparently anomalous finding is the fact that $uptake_1$ is inhibited during TNS even in the absence of the $uptake_1$ blockers (thus not much further effect would be expected when the blockers are introduced) and in the case of cocaine its local anaesthetic effects.

Although the evidence for pre-snyaptic α-receptor mediated inhibition of noradrenaline appears strong on the above evidence, its existence has been questioned by Kalsner(180) resulting in lively discussions via journal articles with the proponents of the theory(181). The evidence against the theory is summarised below.

1. Angus and Korner(182) investigated the effects of α-antagonists on the chronotropic response of guinea pig atria to a single pulse or a train four pulses (one pulse following each of four consecutive atrial electrograms) applied during the refractory period. Under control conditions a tachycardia was observed. Although phenoxybenzamine produced a marked potentiation of this response, two other α-receptor antagonists, phentolamine and yohimbine were without effect on the response to both single and four pulse stimulation. The same result was obtained when the four pulses were applied within one refractory period. As the concentrations of phentolamine and yohimbine used were adequate for effective blockade of α-receptors, and as yohimbine is known to be a relatively selective α_2-antagonist, these findings are unexplainable on the basis of the pre-synaptic α-receptor theory. The uptake blockers desipramine and β-oestradiol (used in combination) produced a similar potentiation to phenoxybenzamine in this prepararation. This suggests that the potentiation produced by phenoxybenzamine may have resulted from uptake blockade rather than pre-synaptic α-receptor blockade in this preparation. Thus, no evidence was available for pre-synaptic inhibition in the guinea-pig atrium during the experimental conditions of the above study. Rand and co-workers(181) in a separate study investigated the effects of four pulses at frequencies of 0.125, 0.25, 0.5, 1 and 2Hz in the guinea-pig atrium measuring the overflow of ^{3}H-noradrenaline. Phentolamine increased the efflux and the cardiac responses at 0.25, 0.5 and 1 Hz but not a 0.125 or 2Hz. The conclusion

reached was that the pre-synaptic inhibitory mechanism had a latency in excess of 1.5 seconds (as the inhibitory feedback was not present at 2 Hz) and had a persistence of between 4 and 8 seconds (as the inhibitory effect is present at 0.25 Hz but not at 0.125 Hz(181). It was further postulated that a mechanism with such temporal characteristics would probably involve a second-messenger formation and destruction, with the pre-junctional receptor probably coupled to an enzyme generating the second messenger(181). However, it should be noted that there is no evidence available at the present time for any of the above conclusions reached by Rand, McCullogh and Story.

2. Kalsner(183) investigated the effect of phenoxybenzamine (3.3 x 10^{-5} mol/1) on the efflux of ^{3}H-noradrenaline and the mechanical response of the guinea-pig vas deferens to a single pulse and a train of four pulses at 5Hz. Phenoxybenzamine increased the efflux of ^{3}H-noradrenaline in response to a single pulse to 328 per cent its control value. The mechanical response was also increased four-fold. A similar result was obtained with the four-pulse train. Blockade of both uptake$_1$ and uptake$_2$ did not significantly affect the mechanical response or the efflux to a single pulse. The potentiating effects of phenoxybenzamine was still evident even after this uptake blockade. Phenoxybenzamine also increased the basal efflux of ^{3}H-noradrenaline in this preparation. The marked potentiation of the response and efflux to a single pulse cannnot be easily explained on the basis of the pre-synaptic α-receptor theory. The possibility that noradrenaline released initially during a single pulse may

inhibit further release by the same pulse appears unlikely as the inward Ca^{2+} current (which triggers the exocytotic release) is essentially terminated with the end of the action potential: the release itself may not commence for another few milliseconds. Rand and co-workers(181) explained these observations on the basis that spontaneously released transmitter may be exerting a feed-back inhibition in the vas deferens in the resting state: phenoxybenzamine by removing this inhibition would increase the efflux of ^3H-noradrenaline produced by a single pulse. The extremely narrow cleft in the vas deferens(144) was suggested as the factor responsible for the effect of spontaneously released transmitter (as a higher concentration would be attained with a narrow cleft). Although this is plausible no evidence is available at present that such a phenomenon actually takes place. Further, Angus and Korner(182) observed a similar potentiating effect of phenoxybenzamine in the guinea-pig right atrium which has a wider cleft. It could be argued that spontaneously released noradrenaline would still act on the pre-synaptic α-receptors in the guinea-pig right atrium. However, this argument would not be tenable with the previous explanation given by Rand et al for the absence of potentiation by phentolamine of the efflux produced by stimulation at 0.125 Hz: it was suggested that pre-synaptic inhibition had a persistence of less than 8 seconds. Thus, if spontaneously released noradrenaline exerted an inhibitory effect, phentolamine should potentiate the efflux even at a stimulation frequency of 0.125 Hz.

3. If negative-feedback inhibition takes place during continued release of endogenous noradrenaline at the cleft, the magnitude of the inhibition of efflux inhibition, produced by a fixed concentration of exogenous noradrenaline should decline as the synaptic concentration of noradrenaline is increased by increased endogeneous transmitter liberation. However, a fixed concentration of exogenous noradrenaline decreased the stimulation-induced efflux of ^{3}H-noradenaline to 300 pulses delivered at 2,5 and 15 Hz to a similar extent (43, 38 and 44 per cent inhibition respectively at the same three frequencies) in the renal arteries(180). Nevertheless, the contractile response increased in a frequency dependent manner indicating that the synaptic concentration of the transmitter rose with an increasing frequency.

4. Although phenoxybenzamine and dibenamine increased the stimulation induced efflux of ^{3}H-noradrenaline in bovine radial artery strips, yohimbine (an α-antagonist which is believed to be more specific for the pre-synaptic receptor) decreased the efflux. Pretreatment of tissues with phenoxybenzamine did not diminish yohimbine inhibition, ruling out action at the same site as a partial agonist(184). Phentolamine tested in the renal arteries failed to enhance stimulation-induced efflux over a frequency of 1-15 Hz.

5. A lack of correlation between the pre-synaptic inhibition of noradrenaline release and the end organ responses during TNS has been observed(180,185). This is, of course, complicated by the fact that the α-agonists and α-antagonists used in efflux

experiments would exert post-junctional effects by themselves. Utilisation of specific α-agonists and α_2-antagonists will not overcome this confounding effect as α_2-receptors have been discovered post-junctionally as well, during the last few years.

Kalsner regards the observation of lesser pre-synaptic inhibition with increasing frequency of stimulation as being against the theory(180). It was suggested that higher cleft concentrations produced by higher frequencies of stimulation should lead to a greater effect on the pre-synaptic α_2-receptors. However, this argument cannot be accepted as evidence against the theory as it is only a philosophical argument that regards the pre-synaptic inhibition as the dominant event at the adrenergic neural cleft. It could be argued equally well that higher frequencies of stimulation should be able to override pre-synaptic inhibition, as the prime function at the cleft should be the release of transmitter.

Another factor that has to be considered in experiments investigating pre-synaptic modulation is the magnitudes of the increases or decreases in transmitter release observed. In some experiments the increases or the decreases in transmitter release amount to less than single fold changes. Although a single fold change may seem substantial a typical dose-response may span a 10,000 fold concentration range. Thus, a single-fold change in transmitter release may not have any appreciable effect on the effector response(180). Although this by itself does not argue against the existence of a pre-synaptic theory it questions the significance of the effects of pre-synaptic inhibition with respect to the end-organ responses. However, it should be noted that much larger changes in transmitter release have been observed in many experiments(186).

In summary, although considerable evidence is available at present for the pre-synaptic α_2-receptor theory, a number of observations remain unexplainable on the basis of this theory. The significance of the phenomenon may differ markedly not only between species and between tissues, but also with the particular experimental condition being studied. Thus, caution should be exercised in using this theory to explain experimental observations unless the existence of the phenomenon has been demonstrated in the particular tissue studied under similar experimental situations.

In addition to the pre-synaptic α_2-receptors, a variety of other pre-synaptic receptors have been described at adrenergic nerve endings(15,129,171,179). Some, like the pre-synaptic α_2-receptors, have been shown to inhibit the release of noradrenaline while others have been shown to facilitate the release of the transmitter. The inhibitory mediators include acetylcholine(187,188,189), adenosine and adenine nucleotides(190,191), histamine (H_2), 5-hydroxytryptamine(192) and some prostaglandins(193,194,195). The facilitatory substances include angiotensin(196), β-agonists(197,198) and some prostaglandins(193). The effects of all these substances are species and tissue dependent as in the case of pre-synaptic α_2 effects(15,129,164,171,179).

METHODS

GENERAL METHODS

Lateral saphenous veins excised from anaesthetised mongrel dogs (15-25 kg body weight) of either sex were utilised in the studies. The dogs were anaesthetised with sodium pentobarbital (Somnotol) or α-chloralose (100 mg/kg body weight) and the veins excised together with the surrounding fatty and connective tissue. Care was taken during dissection to avoid unnecessary stretching or trauma to the veins from the instruments used for the excision. The excised vein segments were immediately placed in oxygenated, cold, modified Krebs-bicarbonate buffer solution of the following composition (mmol/l): NaCl 116.0, KCl 5.4, $CaCl_2$ 1.2, $NaHCO_3$ 22.0, NaH_2PO_4 1.2, $MgCl_2.6H_2O$ 1.2, $CaNa_2EDTA$ 0.023).

Preparation of venous strips and rings(199)

The excised veins were placed in a dissecting tray containing cold Krebs-bicarbonate buffer solution and the excess fatty and connective tissue removed while leaving the thin, nearly transparent sheath of connective tissue surrounding the tunica adventitia intact. Preserving this sheath helped in obtaining a maximum response to transmural nerve stimulation closer to the maximum contraction produced by exogenous noradrenaline. This is possibly due to the fact that the nerves innervating the tunica media of the blood vessels course along the surface of the blood vessel before extending into the media through the adventitia. The blood vessels were then cut into either spiral strips or rings. The spiral strips were cut at an angle of approximately 45° to the long axis of the blood vessels using a pair of fine microdissection scissors. They were approximately 2.0 - 2.5 mm in width and 15 - 20 mm in length. A strip cut properly tended to curl itself back into the shape of an intact blood vessel. The rings (approximately 4 mm in width) were prepared by cutting transversely across the venous segments with a pair of sharp scissors or a razor blade. During this whole procedure (of cutting strips or rings) the tissue was kept moistened with oxygenated Krebs-bicarbonate solution.

The strips and rings were suspended in tissue baths (capacity 12 ml or 22 ml) containing Krebs-bicarbonate buffer solution at a pH of 7.4. The apparatus used is shown in Fig. 11. The buffer solution was maintained at 37°C and continuously aerated with a gas mixture containing 95 per cent O_2 and 5 per cent CO_2, the flow rate of which was controlled by an aerator valve. The temperature in the tissue bath fluid was maintained constant with the aid of a heater/circulater (Model No. E15, Haake Mess-Technik Gmbt U.Co., Karlshrue, Federal Republic of Germany) which circulated heated water through a water jacket incorporated in the tissue bath. Fresh Krebs-bicarbonate buffer solution was fed into the tissue bath from a reservoir which was also aerated and maintained at 37°C. The bath fluid was changed with the aid of drainage/"feeder" tubes and the bath volume was maintained constant by means of an overflow tube. Both drainage and overflow tubes were connected to a vacuum apparatus to ensure complete drainage.

The strips were mounted between two parallel, rectangular, platinum electrodes and their lower ends attached to a moveable support, allowing fine adjustments in the lengths of the strips. The upper ends of the strips were connected to a force transducer (Model No. FT .03C Grass Instrument Co., Quincy, Mass. U.S.A.) for isometric tension recording. The outputs of the transducers were amplified and recorded (Model No.

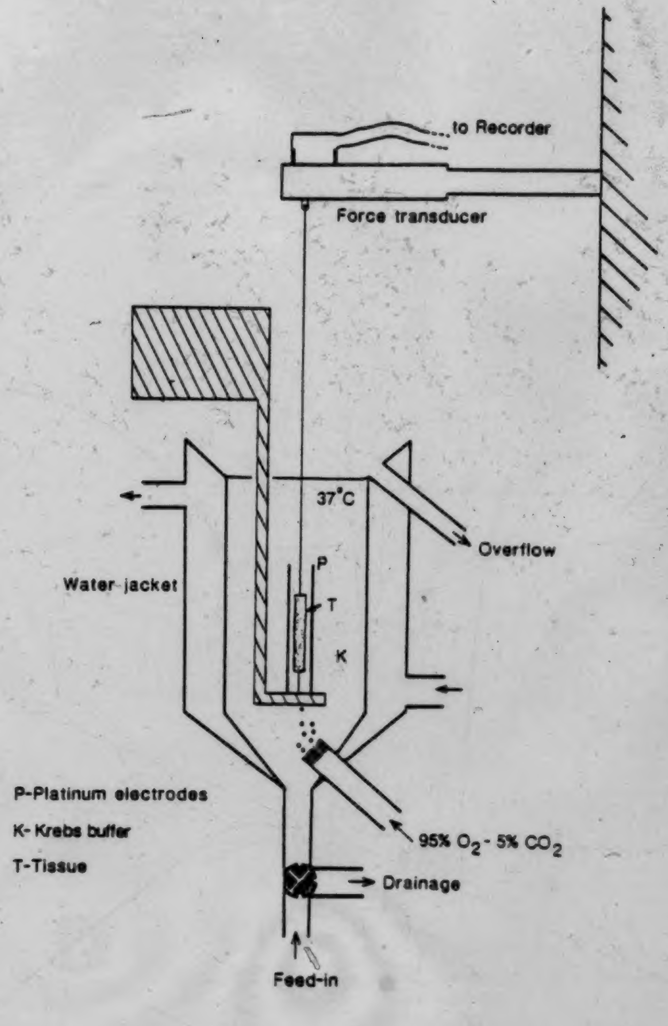

to Recorder
Force transducer
37°C
Overflow
P
Water-jacket
T
K
P-Platinum electrodes
K-Krebs buffer
T-Tissue
95% O_2 - 5% CO_2
Drainage
Feed-in

Figure 11. A diagrammatic representation of the apparatus used for the pharmacological experiments on the isolated canine saphenous vein in the present study.

2400S, Gould Inc. Cleveland, Ohio, U.S.A.). When rings were used they were mounted on two stainless steel triangular clips and the triangular clips in turn attached to the moveable support at the lower end and the force transducer at the upper end. 5-0 silk string was used for all attachments. Transmural nerve stimulation (TNS) was applied from a stimulator via an impedance coupler(200). The electrical impulses consisted of square wave pulses: 1.0 ms duration, 10 volts. With the impedance coupler the voltage measured across the platinum electrodes was approximately equal to the voltage setting on the stimulator. The duration of the square wave pulses as well as the voltage across the electrodes were periodically monitored with the aid of an oscilloscope, to ensure that these values corresponded to the dial settings on the stimulator. Before experimentation, the rings were stretched to the optimum points of their length-tension curves using the method described by Vanhoutte and Leusen(201). This involved the determination of the length at which the response to a fixed train of electrical stimuli is a maximum (train parameters; 10 volts, 1.0 ms, 16 Hz for 10 seconds). The length of the preparation was increased (1 mm every 3 minutes) starting from a length at which the tension was approximately zero. Following 2 minutes of stabilisation at each length the preparation was stimulated with the standard train of stimuli. With the initial increases in length the response to the train of stimuli increased. The magnitude of the response reached a plateau with further increases in length and then decreased if lengthening was continued further. The optimum length was regarded as the length at which magnitude of the responses reached a plateau. The preparations were then equilibrated for a further 90 minutes before the experiment proper. During this time the bath fluid was replaced with fresh buffer solution every 30 minutes.

Dose-response curves to all agonists were obtained in a cumulative manner, graded doses being added to give the desired concentration of the drug in the tissue bath(202). The total volume of solution added to the tissue bath during a cumulative dose-response curve was less than 4 per cent of the total volume of the bath. Following a complete dose-response curve no other drug was tested for at least 60 minutes with repeated changes in the bath fluid during this period, to minimise any adverse effects due to desensitisation.

Stimulus-response curves to transmural nerve stimulation was obtained in a cumulative manner, by increasing the frequency two-fold at each step from 0.5 through 16 Hz (pulse duration 1.0 ms, 10 volts).

SPECIFIC METHODS

The specific protocols carried out are listed below:

Interaction Experiments: Protocol One

Protocol 1.1

Determination of the response to exogenous noradrenaline against a background contraction produced by transmural nerve stimulation (TNS)

Protocol 1.2

Determination of the response to TNS against a background contraction produced by exogenous noradrenaline.

Protocol 1.3

Determination of the response to exogenous noradrenaline against a background electrical current produced by TNS following blockade of the contractile effects of TNS with guanethidine or diltiazem.

Protocol 1.4

Determination of the response to exogenous noradrenaline against a background contraction produced by TNS and against a background contraction produced by either tyramine, methoxamine, histamine or phenylephrine.

Protocol 1.5

Determination of the response to exogeneous noradrenaline against a background contraction produced by TNS and repetition of the same in the presence of either propranalol, indomethacin, aminophylline or cimetidine.

Superfusion Experiments: Protocol Two

Protocol 2.1

Determination of the effect of exogenous noradrenaline applied against a background contraction produced by TNS, on the release of tritiated noradrenaline and its metabolites produced by the TNS: experiment carried out in the presence of cocaine.

Protocol 2.2

Determination of the effect of a background exogenous noradrenaline, on the release of tritiated noradrenaline and its metabolites during TNS applied against this background: experiment carried out in the presence of cocaine.

Protocol 2.3

Column chromatographic analysis of the superfusate samples from Protocol 2.1 and Protcol 2.2 to separate the total radioactivity present in the superfusate into noradrenaline and its metabolites.

Relaxation Experiments: Protocol Three

Protcol 3.1

Determination of the effect of TNS on a vein pre-contracted with prostaglandin $F_{2\alpha}$ following blockade of the contractile response to TNS with guanethidine and phenoxybenzamine.

Protocol 3.2

Determination of the effect of the following drugs on the relaxatory response to TNS observed in Protocol 3.1: cimetidine, indomethacin, ouabain, aminophylline, cyproheptadine, tetrodo toxin, ascorbic acid, catalase.

Protocol 3.3

Determination of the effect of storage of the saphenous veins at 4°C for 9 days, on the relaxatory response to TNS observed in Protocol 3.1.

Protocol 3.4

Determination of the effect of chemical sympathectomy of the saphenous vein rings using 6-hydroxydopamine, on the relaxatory response to TNS observed in Protocol 3.1

Interaction Experiments: Protocol One

In this series of experiments the interaction between transmural nerve stimulation (TNS) and exogenous noradrenaline was studied in isolated canine saphenous vein strips. After setting up of the preparation and equilibration for 90 minutes the following protocols were carried out. Only one protocol was done in each preparation.

Protocol 1.1

This was designed to compare the response to a concentration of exogenous noradrenaline alone, with the response to the same concentration of exogenous noradrenaline added against a background contraction produced by TNS. The protocol consisted of the following steps (Fig. 12). Firstly, a dose-response curve to noradrenaline was obtained: Step A. Tension was then allowed to return to baseline with repeated changes of the bath fluid. This was followed by a stimulus response curve to transmural nerve stimulation: Step B (approximately 60 minutes between completion of dose-response curve in step A and commencement of Step B). Then a dose of noradrenaline was added to produce a contraction between 20-80 per cent of the maximum: Step C. This was taken as the initial control. After washing and equilibration TNS was applied to produce a contraction betweeen 10-90 per cent of the control. Once this contraction reached a plateau the control dose of noradrenaline was added while maintaining the background TNS and the response determined: Step D. After washing and equilibration, the control was repeated: Step E. This was taken as the final control. Steps C, D and E of the protocol were then repeated using different magnitudes of contraction produced by background TNS (range: 10-90 per cent of the control) and different concentrations of exogenous noradrenaline as the control (range: to produce between 20-80 per cent of the maximum contraction to noradrenaline in the preparation).

The study was designed to compare the contraction produced by a concentration of noradrenaline with the additional contraction produced by the same concentration of noradrenaline against a background of TNS. However, a direct comparison between the two would be erroneous for the following reasons. The contractions to noradrenaline in Step C and Step E (controls) and in Step D do not commence from the same level of active tension in the vein. Active tension is approximately zero in

STEP A - NORADRENALINE (NA) DOSE-RESPONSE CURVE
CONCENTRATIONS: 10^{-7} - 10^{-5} MOL/L

STEP B - TRANSMURAL NERVE STIMULATION (TNS) - RESPONSE CURVE
STIMULUS PARAMETERS: 10V, 1 M.SEC, 0.5 - 16 HZ

STEP C - ADDED A DOSE OF NA TO PRODUCE A CONTRACTION BETWEEN 20-80% OF THE MAXIMUM - INITIAL CONTROL

STEP D - APPLIED TNS TO PRODUCE A CONTRACTION LESS THAN INITIAL CONTROL (RANGE 10-90%). CONTROL DOSE OF NA ADDED WHEN CONTRACTION REACHED A PLATEAU. TNS MAINTAINED THROUGHOUT. EFFECT OF NA MEASURED - TEST VALUE

STEP E - STEP C REPEATED - FINAL CONTROL

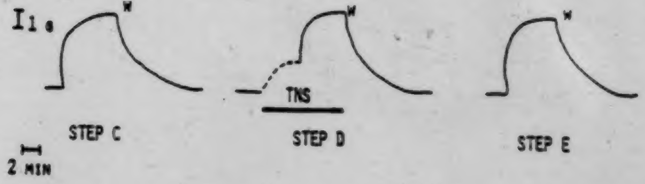


Figure 12. Summary of the steps in Protocol 1.1; NA:exogenous noradrenaline, TNS:transmural nerve stimulation.

Step C and Step E while it is greater than zero by the amount of active tension produced by TNS in Step D. The magnitude of the contraction produced by a fixed amount of an agonist would decrease progressively as the active tension is increased commencing from zero. This is evident from the hyperbolic nature of the dose-response curve to agonists, i.e., a decrease in slope of the dose-response curve as one proceeds along the x-axis. (Note: The dose-response curves to agonists is linear in the mid-range only after a logarithmic transformation of the concentration of the agonist). Thus, the amount of an agonist that is required to produce a contraction from zero to 10 per cent would be much less than the amount needed to increase the contraction from 10 to 20 per cent. Conversely, the magnitude of the contraction produced by a fixed amount of an agonist would lessen as one proceeds along the x-axis of the dose-response curve. This is identical to the situation encountered in Protocol 1.1. Thus a direct comparison between the contractions produced by noradrenaline in Step C and Step E and that produced in Step D is not possible. Before a comparison could be made the magnitude of the contraction that would be produced by the control dose of noradrenaline commencing from an amount of active tension equivalent to that produced by TNS would have to be calculated. This was done as follows (Fig. 13). If the contraction produced by TNS in Step D was X per cent of the maximum (for noradrenaline in the particular preparation studied) and the total contraction produced by TNS and noradrenaline was Y per cent, the observed contraction to the noradrenaline alone would be (Y-X per cent). The expected contraction to the same dose of noradrenaline commencing from an increased level of active tension equivalent to that produced by TNS, was calculated using the

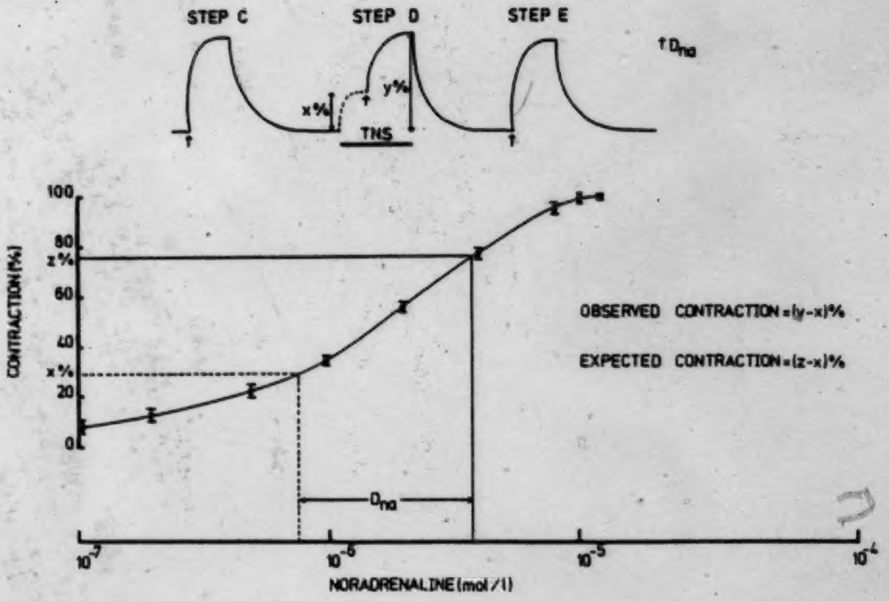


Figure 13. A diagrammatic representation of the method of calculation of the expected contraction for a fixed concentration of exogenous noradrenaline (D_{na}), added against a background contraction produced by transmural nerve stimulation (TNS) in Protocol 1.1. Step C, Step D and Step E refer to the respective steps in Protocol 1.1 (refer Fig. 12). All percentages have been expressed taking the maximum contraction to exogenous noradrenaline in the canine saphenous vein as a 100 per cent.

noradrenaline dose-response curve shown in Fig. 13. The X per cent contraction produced by the background TNS was related to a notional point on the abscissa. The same concentration of noradrenaline as used in the controls was added along the abscissa starting from this point and the corresponding total contraction was read off the ordinate. This is represented by Z per cent. Thus, the expected contraction was taken as (Z-X per cent). Comparisons were made between the observed contractions and the expected contractions using the student's t-test for paired data. The dose-response curve used for calculating the expected contractions was obtained by pooling the data from the dose-response curves done each day. Although this method of determining an expected contraction may not be without error, it was used as the best method available as a direct comparison would be meaningless as a result of the situation described above.

Protocol 1.2

This was designed to compare the response to a frequency of transmural nerve stimulation with the response to the same frequency of TNS against a background contraction produced by exogenous noradrenaline, i.e., the reciprocal of Protocol 1.1. The protocol consisted of the following steps (Fig. 14). Firstly, a dose-response curve to noradrenaline was done: Step A. This was followed by a stimulus-response curve to transmural nerve stimulation: Step B. Then a frequency of TNS was applied to produced a contraction between 20-80 per cent of the maximum: Step C. This was taken as the initial control. After washing and equilibration exogenous noradrenaline was added to the tissue bath to produce a contraction between 10-90 per cent of the control. Once this contraction reached a plateau the control frequency

STEP A - NORADRENALINE(NA) DOSE-RESPONSE CURVE.
CONCENTRATIONS: 10^{-7} - 10^{-5} MOL/L

STEP B - TRANSMURAL NERVE STIMULATION (TNS)-RESPONSE CURVE
STIMULUS PARAMETERS: 10V, 1 M.SEC, 0.5-16 HZ

STEP C - APPLIED TNS TO PRODUCE A CONTRACTION BETWEEN 20-80% OF THE MAXIMUM - INITIAL CONTROL

STEP D - ADDED NA TO PRODUCE A CONTRACTION LESS THAN INITIAL CONTROL (RANGE 10-90%). WHEN CONTRACTION REACHED A PLATEAU, CONTROL TNS APPLIED. NA PRESENT IN BATH THROUGHOUT. EFFECT OF TNS MEASURED - TEST VALUE

STEP E - STEP C REPEATED - FINAL CONTROL

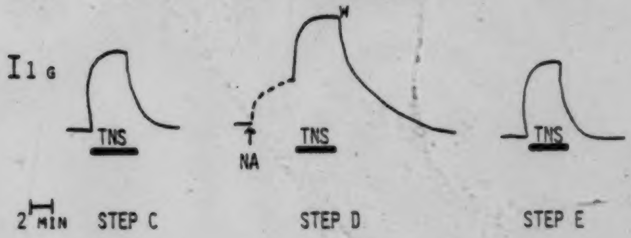


Figure 14. Summary of the steps in Protocol 1.2; NA:exogenous noradrenaline, TNS:transmural nerve stimulation.

of TNS was applied and the response determined while the exogenous noradrenaline remained in the tissue bath: Step D. After washing and equilibration the control was repeated: Step E. Steps C, D and E of the protocol were then repeated using different magnitudes of contraction produced by the background exogenous noradrenaline (range: 10-90 per cent of control) and different frequencies of TNS as the control (range: to produce 20-80 per cent of the maximum contraction to noradrenaline in the preparation). The frequencies of TNS and the concentrations of exogenous noradrenaline in Step D of both protocols were chosen in a manner that the total contraction in Step D would be less than the maximum obtainable with exogenous noradrenaline alone in the saphenous vein. This ensured that the total response observed in Step D was not limited by the maximum contraction attainable to noradrenaline in the preparation.

Protocol 2.2 was designed to compare the contraction produced by a frequency of TNS with the additional contraction produced by the same frequency of TNS against a background contraction produced by exogenous noradrenaline. As detailed under Protocol 1.1 a direct comparison would be erroneous as the contraction to TNS in Step C and Step E (controls) and in Step D do not commence from the same level of active tension. Thus, before a comparison could be made the magnitude of the contraction that would be produced by the control frequency of TNS commencing from an amount of active tension equivalent to that produced by exogenous noradrenaline would have to be calculated. This was done as follows (Fig. 15). If the contraction produced by TNS alone in the controls is C per cent of the maximum (for noradrenaline in the particular preparation studied) this could be related to a notional concentration

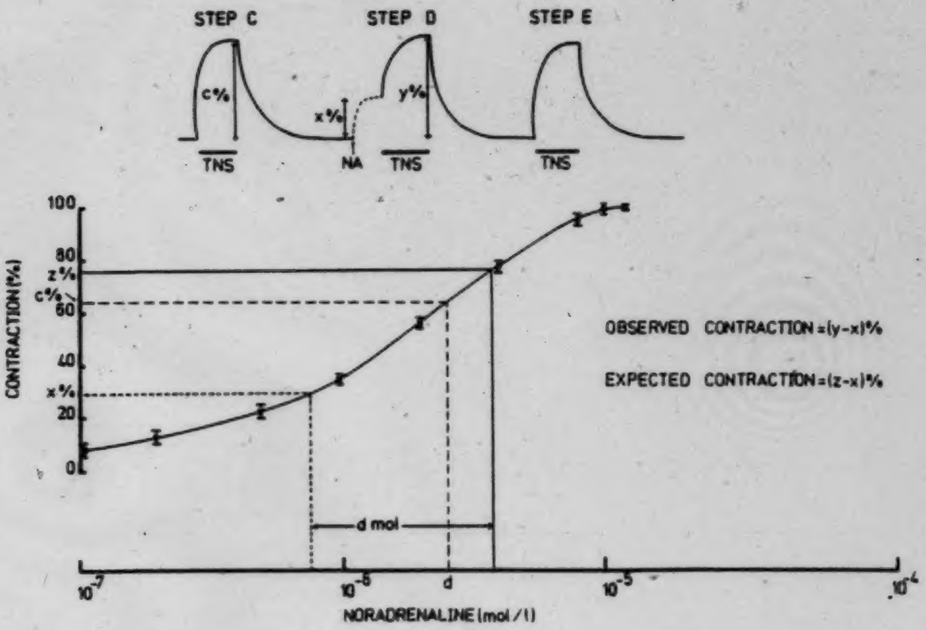

STEP C
STEP D
STEP E
c%
x%
y%
TNS
NA
TNS
TNS
100
80
z%
c%
60
40
x%
20
0
CONTRACTION (%)
OBSERVED CONTRACTION = (y-x)%
EXPECTED CONTRACTION = (z-x)%
d mol
10^{-7}
10^{-6}
d
10^{-5}
10^{-4}
NORADRENALINE (mol / l)

Figure 15. A diagrammatic representation of the method of calculation of the expected contraction for a fixed frequency of transmural nerve stimulation (TNS), applied against a background contraction produced by exogenous noradrenaline (NA) in Protocol 1.2. Step C, Step D, and Step E refer to the steps in Protocol 1.2 (refer Fig. 14). All percentages have been expressed, considering the maximum contraction to exogenous noradrenaline in the canine saphenous vein as a 100 percent.

of noradrenaline on the abscissa (represented by d mol/l). This would be the concentration of exogenous noradrenaline necessary to produce a contraction equivalent to that produced by the control frequency of TNS, and was used as a substitute for the latter in calculating the *expected contractions.* If the contraction produced by exogenous noradrenaline in Step D was X per cent of the maximum (for noradrenaline in the particular preparation studied) and the total contraction produced by the exogenous noradrenaline and TNS was Y per cent the *observed contraction* to the TNS alone would be (Y-X per cent). The expected contraction to the same frequency of TNS commencing from a level of active tension equivalent to that produced by the exogenous noradrenaline was calculated using the noradrenaline dose-response curve shown in Fig. 15. The X per cent contraction produced by the background noradrenaline was related to a notional point along the abscissa. The concentration of exogenous noradrenaline that was equivalent to the control frequency of TNS was added along the abscissa starting from this notional point and the corresponding total contraction read off the ordinate. This is represented by Z per cent. Thus, the *expected contraction* was taken as (Z-X per cent). Comparisons were made between the *observed contractions* and *expected contractions* using the student's t-test for paired data. The dose-response curve used for calculating the *expected contractions* was obtained by pooling the data from the individual dose-response curves done each day.

Results of Protocol 1.1 and Protocol 1.2 indicated that the contractions produced by exogenous noradrenaline in the isolated canine saphenous vein were "inhibited" by background TNS as in Protocol 1.1. Therefore further experiments were performed to elucidate the mechanism responsible for this inhibitory phenomenon. These experimental protocols were further extensions and modifications of Protocol 1.1 as the inhibitory phenomenon was evident in this protocol only.

Protocol 1.3

This protocol was performed to determine whether the inhibitory effect of TNS on the exogenous noradrenaline contraction was due to the excitation of intramural nerves or due to the field of current per se. Steps A-E of Protocol 1.1 were done as before. Then the adrenergic neurone blocking agent guanethidine was added to the tissue bath in a concentration just sufficient to block the contractile effects of TNS at the frequency used in Step D. (Note: Complete abolition of the contractile effects of TNS up to a frequency of 32 Hz was not attempted). Guanethidine was kept in the tissue bath for a minimum of 60 minutes before further experiments were carried out and was present in the bath throughout the rest of the experiment. After the blockade of the contractile response to TNS, Steps C, D and E were repeated i.e., the two controls and Step D. In Step D, TNS, at the same frequency as used before the addition of guanethidine, was applied first. This did not produce a response but the flow of electrical current between the platinum electrodes during the current pulses would still be present. After 5 minutes of TNS the control dose of noradrenaline was added while maintaining the current and the response determined. The contraction produced by exogenous noradrenaline in Step D was compared with the mean of that produced in Step C and Step E by a student's t-test for paired data. Calculation of expected contractions to noradrenaline was not necessary as the contractions with and without guanethidine commenced from the same level of active tension (which was approximately zero in the present instance).

Steps C, D, and E of the protocol were also repeated using the calcium channel blocker diltiazam in place of guanethidine. Diltiazem produced a differential effect on the contractions produced by TNS as compared with the contractions produced by exogenous noradrenaline in the canine saphenous vein. It was used to block the contractile effects of TNS while leaving the contractile effects of exogenous noradrenaline relatively unaltered. Again, complete blockade of TNS induced contractions up to a frequency of 32 Hz was not attempted. A concentration sufficient to block the effects of TNS at the frequency used in Step D was used. Incubations were carried out for minimum of 60 minutes with diltiazem and it was present in the bath fluid for the rest of the experiment.

Protocol 1.4

These experiments were designed to determine whether the inhibitory effect of background TNS, on the exogenous noradrenaline mediated contraction, observed in Protocol 1.1 was specific for TNS or whether it would still be evident when another agonist is substituted in place of TNS. The agonists used were:

(a) tyramine - an indirectly acting sympathomimetic agent

(b) methoxamine-a specific α_1 agonist which is a poor substrate for uptake$_1$ and thus with no indirect sympathomimetic effects

(c) histamine

(d) phenylephrine

The concentration of tyramine used was kept low enough to avoid direct effects on smooth muscle which appear at higher concentrations. The concentration range which produced indirect effects only was determined in preliminary experiments where dose-response curves to tyramine were done with and without an uptake_1 inhibitor.

In Protocol 1.4, Steps A-E of Protocol 1.1 were done first. Then a dose of tyramine (or methoxamine or histamine) was added into the tissue bath to produce a contraction approximately equal in magnitude to that produced by the background TNS in Step D (Fig. 16). Once this contraction to tyramine reached a plateau, the control dose of noradrenaline was added and the response determined. The additional contraction produced by noradrenaline against a background of tyramine was compared with the additional contraction produced by noradrenaline against a background of TNS with the student's t-test for paired data. Calculation of expected contractions to noradrenaline was not necessary as the magnitude of the background tensions (produced by TNS and tyramine) were approximately equal. In practice the triad (steps C, D and E) with tyramine (or histamine or methoxamine) was carried out first and then the triad was repeated substituting TNS in place of tyramine as it was easier to match a contraction by TNS equal in magnitude to that produced by tyramine than vice versa.

Protocol 1.5

This was designed to investigate whether the inhibitory effect of TNS on the exogenous noradrenaline mediated contraction observed in Protocol 1.1 would still be present in the presence of the

a) beta-blocker propranolol (10^{-5} mol/l)
b) cyclo-oxygenase inhibitor indomethacin (10^{-5} mol/l)
c) P_1-purinoceptor antagonist aminophylline (10^{-5}mol/l)
d) H_2-receptor antagonist cimetidine 10^{-4} mol/l

Firstly, steps A-E of Protocol 1.1 were repeated as before. Then one of the above drugs was added to the tissue bath to produce the

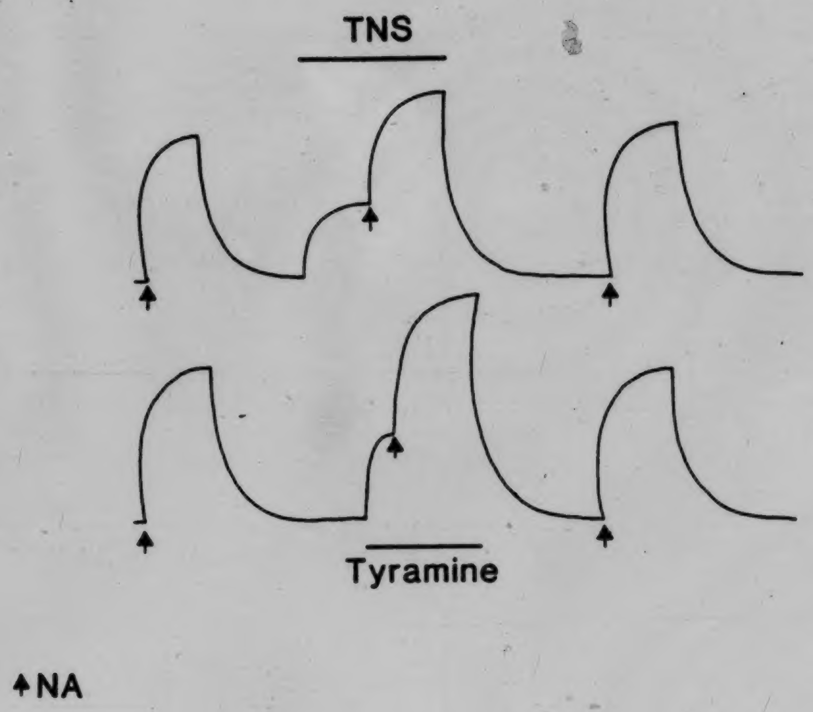


Figure 16. Steps of Protocol 1.4. The first part of the protocol is shown in the upper half of the figure: the control contraction to the exogenous noradrenaline (initial control), the contraction to the same concentration of noradrenaline added against a background contraction produced by transmural nerve stimulation (TNS) and the final control. The second part of the protocol is shown in the lower half of the figure: the initial control, the contraction to the same concentration of noradrenaline added against a background contraction by tyramine (of equal magnitude to the contraction produced by TNS in the first part of the study) and the final control.

concentration given above. The drug was then present in the bath for the rest of the experiment. Steps C,D, and E were repeated following incubation with the drug for a minimum of 30 minutes. Only one drug was tested in one vein strip. The additional contractions produced by exogenous noradrenaline against a background of TNS with and without each drug were compared with the student's t-test for paired data.

Superfusion Experiments: Protocol Two

In step D of Protocol 1.1 exogenous noradrenaline was added against a background contraction produced by TNS and the additional response measured. When the additional response is taken as that produced by the exogenous noradrenaline, the assumption made is that the magnitude of the contraction produced by the background TNS remains unchanged during this period. However, exogenous noradrenaline is known to produce inhibition of release of noradrenaline by (thus inhibition of the contraction by) the sympathetic nerves acting on pre-synaptic α_2 receptors(203). If such a phenomenon takes place in Step D of Protocol 1.1 this could confound the results. A similar situation could take place in Step D of Protocol 1.2 when TNS is applied against a background contraction produced by exogenous noradrenaline. Thus it was decided to investigate these two confounding effects by measuring the release of noradrenaline from the sympathetic nerves in isolated canine saphenous veins during experimental situations similar to Protocol 1.1 and Protocol 1.2.

A modification of the superfusion technique described by Vanhoutte et al(188) using tritiated noradrenaline was utilised for this study. Lateral saphenous veins were isolated from anaesthetised dogs as described under General Methods. The veins were cut into spiral strips approximately 2-3 mm in diameter and 50-60 mm in length. These preparations were incubated in 8 ml of Krebs buffer solution containing 7-^{3}H-noradrenaline (specific activity 10-30 Ci/mmol) in a concentration of 10^{-6} mol/l while aerating the solution with 95 per cent O_2 -5 per cent CO_2. This was carried out in a fumehood. After 60 minutes the strips were then transferred to a fresh solution of ^{3}H-noradrenaline in Krebs solution and incubated for a further 60 minutes. At the end of the second period of incubation the vein strips were rinsed in 25 ml of Krebs buffer solution (without any ^{3}H-noradrenaline) and mounted for superfusion in a glass chamber similar to that described by Hughes and Roth(204) as illustrated in Fig. 17. The strips were mounted between two platinum wires (0.032 cm in diameter) inside a funnel shaped chamber maintained at 37°C with the aid of a water jacket. The platinum wires were used as electrodes by connecting them to the stimulator-impedance coupler system (refer General Methods). The gaps between the electrodes and the strips were wide enough to allow contraction and relaxation without restraint and yet sufficiently narrow to be filled consistently by part of the superfusate retained by capillary action, thus ensuring continued electrical conductivity. The lower end of the strip was attached to a plastic holder and the upper end connected to a force transducer (Model No. FT .03C, Grass Instrument Company, Quincy, Ma. U.S.A.) for isometric tension recording. The transducer was mounted on a moveable support to allow vertical movement. The connection to the transducer was made with a wettable twine string to aid in the superfusion. The vein strip was superfused with oxygenated Krebs-bicarbonate buffer solution pre-warmed to 37°C. The solution was made to drip along the twine string on to the vein strip between the two

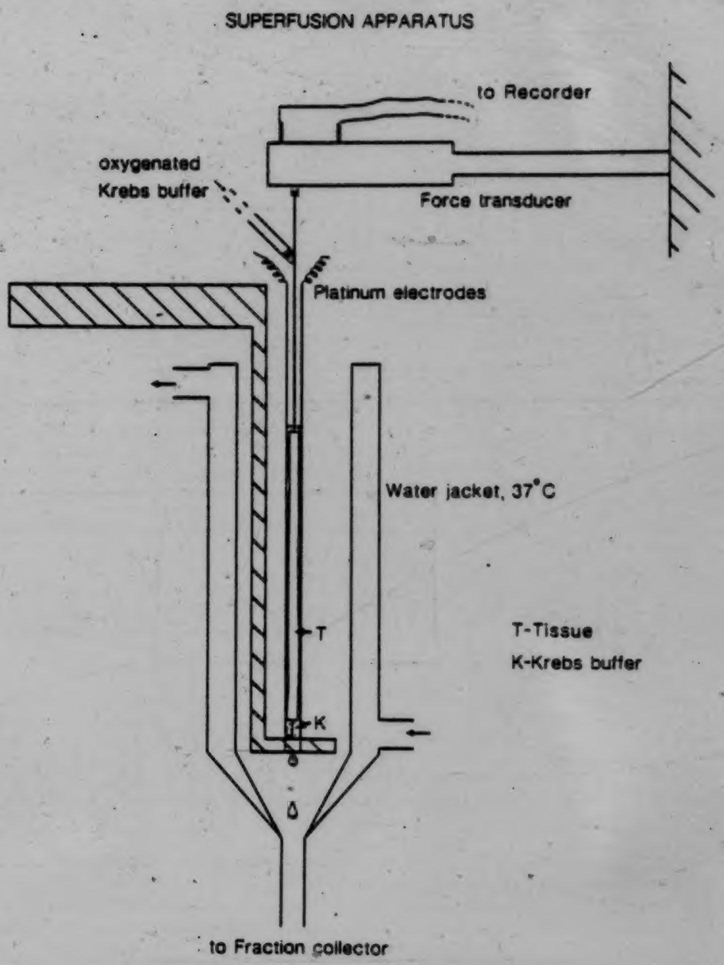

SUPERFUSION APPARATUS
to Recorder
oxygenated Krebs buffer
Force transducer
Platinum electrodes
Water jacket, 37°C
T
K
T-Tissue
K-Krebs buffer
to Fraction collector

Figure 17. A diagrammatic representation of the apparatus used for the superfusion experiments using ^{3}H-noradrenaline in the present study.

platinum electrodes. The buffer solution cascaded over both the vein strip and the electrodes and was collected in test tubes in a fraction collector (Model No. Frac-100, Phrmacia Canada Inc. Dorval, Quebec, Canada) through the outlet tube of the superfusion chamber. The samples were collected on a time-basis with a change of the tubes every 2 minutes. The superfusion was carried out at a constant flow rate of 3.0 ml/min with the help of a positive displacement roller pump (Model No. Miniplus 2, Gilson Medical electronics, Viliers. le.bel., France). A three-way stopcock upstream from the pump allowed rapid switching from control Krebs buffer solution to buffer solutions containing drugs whenever necessary.

After setting up of the vein strips the initial basal tension was set at 3.0g by stretching the strips (by moving the force transducer upwards). Superfusion with Krebs buffer solution was continued for 120 minutes before the experiment proper, to allow for any loosely bound H^3-noradrenaline to be "washed" away and the basal efflux of radioactivity to stabilise at a steady level. The experiments (Protocol 2.1 and Protocol 2.2) were carried out in the presence of cocaine to inhibit $uptake_1$ as pre-synaptic α_2 inhibition has generally been demonstrated in the presence of an $uptake_1$ inhibitor(174). The superfusing solution was changed to Krebs buffer solution containing cocaine (3×10^{-5} mol/l) 30 minutes before the commencement of the experiment.

Protocol 2.1

In order to determine the effects of exogenous noradrenaline on the efflux of ^3H-noradrenaline during TNS, step D of Protocol 1.1 was carried out as a superfusion experiment. As exogenous noradrenaline does not change the basal efflux of ^3H-noradrenaline significantly in the presence of cocaine(174), Step C and Step E Protocol 1.1 (i.e., the measurement of the response to a concentration of exogenous noradrenaline alone) were not done here. The experiment proper was carried out as follows. The numbers refer to the consecutive test tube numbers in the fraction collector (each tube corresponds to a two-minute period of collection containing 2 x 3.0 ml=6.0 ml of the superfusate). The triad of consecutive tubes used for determination of the efflux of ^{3}H-noradrenaline representative for that particular period of the protocol are denoted by asterisks. These constituted the final three tubes of each period. The pooled superfusate from the same triad of tubes was used in column chromatography to separate the total radioactivity into noradrenaline per se and its different metabolites.

PERIOD I: 1* 2* 3* (CONTROL)

Superfusate was collected for 6 minutes (3 tubes) as a control period for determination of the basal efflux ^{3}H-noradrenaline.

PERIOD II: 4 5 6 7 8* 9* 10* (TNS)

TNS was applied at 2 Hz for 14 minutes while superfusing with Krebs buffer solution.

PERIOD III: 11 12 13 14 15* 16* 17* (TNS + NA)

Superfusing solution was changed to Krebs buffer containing noradrenaline (10^{-6} mol/l) for the next 14 minutes while maintaining TNS.

PERIOD IV: 18 19 20 21 22* 23* 24* (TNS)

Superfusing solution was changed back to plain Krebs buffer while maintaining TNS - 14 minutes.

PERIOD V: 25 26 27 28 29 30 31* 32* 33* (CONTROL)

TNS terminated. Superfusion was continued with plain Krebs buffer for the next 18 minutes.

The protocol is summarized in Fig. 18

Protocol 2.1

Tube no.
1*
2*
3* CONTROL
4 TNS
5
6
7
8*
9*
10* TNS
11 TNS + NA
12
13
14
15*
16*
17* TNS + NA
18 TNS
19
20
21
22*
23*
24* TNS
25 CONTROL
26
27
28
29
30
31*
32*
33* CONTROL
END OF EXPERIMENT

Fig. 18. Summary of Protocol 2.1. Numbers refer to consecutive test tube numbers in the fraction collector. Each tube represents a 2 minute period of collection of superfusate. Asterisks denote the tubes utilised for subsequent column chromatographic analysis.

Total radioactivity present in each sample collected (total of 33 tubes) was determined using a 1.0 ml aliquot of the superfusate (details given below). The superfusate collected during selected 6 minute intervals (the three consectuive test tubes indicated by asterisks) was also utilised for subsequent column chromatographic analysis to separate ^{3}H-noradrenaline from its metabolites. To minimise the adsorption of tritiated noradrenaline and its metabolites on to the glass wall of these triads of test tubes, each test tube contained 0.5 ml of a stock carrier solution containing unlabelled noradrenaline and each of its five major metabolites (composition of stock solution given below). The stock carrier solution also contained sodium metabisulphite, disodium ethylene diamine tetraacetic acid and hydrochloric acid as protective agents to prevent oxidation of catecholamines. These tubes were kept in the refrigerator until just prior to collection of the superfusate. Following collection of the superfusate 1.0 ml was pipetted out for total radioactivity measurements as explained above. (A correction was made in the calculation of total radioactivity in each of these samples saved for subsequent column chromatographic analysis as the 6.0 ml of superfusate in each test tube was diluted to 6.5 ml by the addition of 0.5 ml of the stock carrier solution). The remaining superfusate (approximately 5.5 ml in each tube) from the 3 tubes of each triad were pooled together (making up approximately 16.5 ml) and immediately frozen and kept for column chromatographic analysis which was carried out over the following week.

At the end of the experiment the saphenous vein strips were removed from the superfusion chamber for the estimation of the amount of total radioactivity present in each vein strip. The tritiated noradrenaline and metabolites retained in the tissue were extracted(205) by placing each strip in a small vial containing 2.5 ml of extraction fluid (see below for composition) and agitating the contents of the vial continuously for 30 minutes. Then the strip was transferred to another vial containing the same extraction fluid. After a further 30 minutes of agitation, the strips were removed, and the two 2.5 ml extraction portions were pooled and mixed. A 1.0 ml aliquot from this 5.0 ml was used for measurement of the radioactivity present in the tissue.

The amount of radioactivity present in each 2 minute (6 ml) sample collected, was expressed as a fraction of the total radioactivity present in the strip, at that particular time-referred to as Fractional release(206). Thus the radioactivity present in the last sample collected was expressed as a fraction of the sum of the total radioactivity present in the tissue extraction fluid and the radioactivity present in the last sample itself. As the resultant fractional release values were small, each value was multiplied by 10^3 for clarity, and expressed in graphs and tables.

e.g.
radioactivity present in the last sample =400 dpm/ml

radioactivity present in the penultimate sample =600 dpm/ml

total radioactivity in the tissue extract
at the end of the experiment =600,000 dpm/ml

Fractional release for last sample $= \dfrac{(400 \times 6)}{(600{,}000\text{x}5)+(400\text{x}6)}$

Fractional release for penultimate sample $= \dfrac{(600\text{x}6)}{(600{,}000\text{x}5)+(400\text{x}6)+(600 \times 6)}$

Preparation of stock carrier solution (Personal communication, Lorenz RR, Vanoutte PM.)

1. A 0.01 per cent solution of each of the following drugs in 0.01 per cent ascorbic acid was prepared by dissolving 10 mg of each drug in the ascorbic acid solution and making it up to 100 ml: noradrenaline (NA), 3,4-dihydroxyphenyl glycol (DOPEG), 3-methoxy-4-hydroxymandelic acid (VMA), normetanephrine (NMN), 3-methoxy-4-hydroxyphenyl glycol (MOPEG), and 3,4-dihydroxymandelic acid (DOMA).

2. 800 mg sodium metabisulphite and 800 mg di-sodium ethylene diamine tetra acetic acid were weighed out into a beaker and 8.0 ml of each of the solutions prepared above were added in the following order, while stirring continuously with the aid of a magnetic stirrer.

   8 ml NE
   8 ml DOPEG
   8 ml VMA
   8 ml NMN
   8 ml MOPEG
   8 ml DOMA

   8 ml of 5 N hydrochloric acid was added last. The resulting stock carrier "solution" which was a suspension was stored at 4°C and was good for 3-4 weeks.

3. 0.5 ml of the above suspension was added to each of the test tubes constituting the triads of tubes pre-selected for column chromatography. Note: The suspension was stirred well with a

magnetic stirrer and the 0.5 ml was pipetted out while stirring as the drugs are in suspension.

Extraction fluid:Preparation(207)

Extraction fluid used for ^{3}H-noradrenaline extraction from the tissue was made up as follows.

1. 5.5 mg Na_2EDTA and 44.3 mg of ascorbic acid were dissolved in IN acetic acid and the total volume was made up to 500 ml with IN acetic acid itself.
2. The resulting solution was kept at 4°C and was good for 6 months.

Radioactivity measurement

Total radioactivity was measured in all samples collected in the fraction collector. Following the collection, the superfusate contained in each tube (6.0 ml) was mixed thoroughly with the help of a vortex mixer, and a 1.0 ml aliquot pipetted into a liquid scintilation vial. Ten ml of liquid scintilation fluid (Aquasol, New England Nuclear, Canada) was added to each of these vials and mixed vigorously with the 1.0 ml of the superfusate, by shaking to form a clear fluid. Radioactivity was measured in a liquid scintilation counter (Model no. 8500, Beckman Instruments). Each vial was counted for 10 minutes or until 10,000 counts accumulated. Corrections for quenching were made with an external standard and counting efficiency was approximately 36 per cent. The scintillation vials were left for 120 minutes for dark adaptation prior to measurement of radioactivity.

Protocol 2.2

This protocol was carried out to determine the effects of a prior application of exogenous noradrenaline on the efflux of ^{3}H-noradrenaline during transmural nerve stimulation, i.e., corresponding to Protocol 1.2. Thus, Step C, Step D, and Step E of Protocol 1.2 were carried out as a superfusion experiment. Cocaine (3×10^{-5} mol/1) was present in the superfusing Krebs buffer solution throughout the experiment. The experiment proper was carried out as follows. The numbers refer to the consecutive test tubes in the fraction collector. The last three tubes during each period of the protocol were selected for subsequent column chromatographic analysis as in Protocol 2.1. These test tube numbers are denoted by an asterisk.

PERIOD I: 1* 2* 3* (CONTROL)

Superfusate was collected for 6 minutes (three tubes) as a control period for determiantion of the basal efflux of ^{3}H-noradrenaline.

PERIOD II: 4 5 6 7 8* 9* 10* (TNS)

TNS was applied at 3 Hz for 14 minutes while superfusing with plain Krebs buffer solution. TNS was terminated at the end of the 14th minute.

PERIOD III: 11 12 13 14 15 16 17 18 19 (CONTROL)
20 21 22 23 24 25* 26* 27*

Superfusion was continued with plain Krebs buffer solution for 34 minutes

PERIOD IV: 28 29 30 31 32* 33* 34* (NA)

Superfusing solution was changed to Krebs buffer containing noradrenaline (3×10^{-7}mol/1) for the next 14 minutes.

PERIOD V: 35 36 37 38 39* 40* 41* (NA + TNS)
TNS was applied at 3 Hz for 14 minutes while continuing the superfusion with Krebs buffer containing noradrenaline (3.3×10^{-7} mol/1).

PERIOD VI: 42 43 44 45 46 47 48 49 50 51 (CONTROL)
52 53 54 55 56* 57* 58*
TNS was terminated and the superfusing solution changed to plain Krebs buffer solution for the next 34 minutes as a control period.

PERIOD VII: 59 60 61 62 63* 64* 65* (TNS)
TNS was applied at 3 Hz for 14 minutes while superfusing with plain Krebs buffer solution.

PERIOD VIII: 66 67 68 69 70 71 72 73* 74* 75* (CONTROL)
TNS was terminated and the superfusion continued with plain Krebs buffer solution as a control.

At the end of the experiment the total radioactivity remaining in the vein strip was extracted as explained in Protocol 2.1. Total radioactivity was counted in each sample of superfuste collected and column chromatohgraphic analysis was done in selected samples as before. The protocol is summarised in Figure 19.

As stated above, the radioactivity present in the superfusate collected in the triad of test tubes denoted by asterisks were considered as representative for that particular period of the experiment. The last three samples during each period consitituted this triad of test tubes which were also used for the column chromatography. The mean of the radioactivities present in the three samples was used for statistical comparisons.

Protocol 2.2

Tube No.

1*
2*
3* CONTROL
4 TNS
5
6
7
8*
9*
10* TNS
11 CONTROL
12
13
14
15
16
17
18
19
20
21
22
24
25*
26*
27* CONTROL
28 NA
29
30
31
32*
33*
34* NA
35 NA + TNS
36
37
38
39*
40*
41* NA + TNS
42 CONTROL
43
44
45
46
47
48
49
50
52
53
54
55
56*
57*
58* CONTROL
59 TNS
60
61
62*
63*
64*
65 TNS
66 CONTROL
67
68
69
70
71
72
73*
74*
75* CONTROL
END OF EXPERIMENT

Figure 19. Summary of Protocol 2.2. Numbers refer to consecutive test tube numbers in the fraction collector. Each tube represents a 2 minute period of collection of superfusate. Asterisks denote the tubes utilised for subsequent column chromatographic anlysis.

The efflux of ^{3}H-noradrenaline decays with the passage of time in most preparations in superfusion experiments. To correct for this the following procedure was used(174). Evoked release of ^{3}H-noradrenaline during an intervention such as TNS (or a combination of TNS and exogenous noradrenaline) was calculated by subtracting the average basal efflux of ^{3}H-noradrenaline during the two control periods (done before and after the intervention) from the efflux during the intervention.

Column chromatographic analysis(188,208,209)

This was used to separate the radioactive material in the superfusate into noradrenaline (NA) and its major metabolites: 3,4-dihydroxyphenylglycol (DOPEG), 3,4-dihydroxymandelic acid (DOMA), normetanephrine (NMN), 3-methoxy-4-hydroxyphenylglycol (MOPEG) and 3-methoxy-4-hydroxymandelic acid (VMA).

Summary: Alumina, Dowex-50 and Dowex-1 were used for the separation which was carried out in glass columns (0.5 cm in diameter). First, the catechol compounds (NA, DOPEG, DOMA) were separated from the non-catechols by adsorption of the former on alumina. The NA and DOPEG were eluted from the alumina with acetic acid, followed by DOMA which was eluted with 0.2N and IN hydrochloric acid. NA was separated from DOPEG (in the eluted NA/DOPEG-mixture) by adsorption of the former on Dowex-50 and elution later with hydrochloric acid. The non-catechol compounds present in the alumina effluent were separated by adsorption of the NMN in Dowex-50 columns with the effluent containing MOPEG and VMA. The NMN adsorbed on Dowex-50 was subsequently eluted with hydrochloric acid in ethanol. MOPEG and VMA were separated by adsorption of the latter on to Dowex-1 and its subsequent elution with hydrochloric acid.

Aluminium Oxide (Alumina) - washing and activation(210)

This was carried out in a fumehood as follows.

1. 100 g of aluminium oxide was added to 500 ml of 2N hydrochloric acid in a 1000 ml beaker, the beaker covered with a watch glass, and heated at 90° to 100° C for 45 minutes with continuous and rapid stirring, using a magnetic stirrer-hot plate.
2. The beaker was removed from the heater-stirrer and the heavier particles of aluminium oxide allowed to settle for 1 1/2 minutes. The supernatant fluid (distinctly yellow in colour) was discarded along with the finer particles of aluminium oxide.
3. The aluminium oxide was then washed twice with fresh 250 ml portions of 2N hydrochloric acid at 70°C for 10 minutes, discarding the supernatant with the finer aluminum oxide particles each time.
4. Aluminium oxide was washed with 500 ml of 2N hydrochloric acid at 50°C for 10 minutes. Supernatant with the finer alumina particles discarded as before.
5. The alumina was then washed (stirring for 1-10 minutes) repeatedly (about 20-25 times) with fresh 200 ml portions of distilled water until the pH of the suspension was 3.4. The supernatant with the finer particles was decanted each time.
6. Finally, the aluminium oxide was transferred to an evaporating dish and activated by heating at 300°C for 2 hours or at 200°C overnight (A modification of the original method as recommended by Vanhoutte, P.M. and Verbeurn T., personal communication). Alumina was reactivated every week by repeating this step. The alumina was stored in a dessicater at room temperature.

Dowex-50 x 4 resin (200-400 mesh) - washing

1. one volume of the Dowex-50 resin was suspended in approximately three volumes of distilled water and continuously stirred for 20 minutes. The Dowex-50 was then allowed to settle for 30 minutes and the supernatant decanted. This was repeated 4-5 times. Following the final washing the Dowex-50/distilled water suspension was filtered on a Buchner funnel-Buchner flask (with the aid of light negative pressure in the Buchner flask) and air dried overnight.
2. The resin was then suspended in three volumes of 2N hydrochloric acid and continuously stirred for 30 minutes. The suspension was allowed to settle for 30 minutes and the supernatant discarded. This step was repeated with fresh 2N hydrochloric acid until the supernatant was almost colourless - approximately 2-3 times. (This procedure cycles the Dowex - 50 resin through a H^+ form).
3. The resin was suspended in three volumes of distilled water and stirred for 30 minutes, allowed to settle for 30 minutes, and the supernatant discarded. This was repeated once.
4. The Dowex-50 was then suspended in three volumes of 2N sodium hydroxide and stirred for 30 minutes and the supernatant discarded (This cycles the resin through a Na^+ form).
5. The resin was then washed four times with distilled water as in step three, and filtered on a Buchner funnel-flask and allowed to air-dry.
6. The washed resin was stored in a bottle at 4°C.

Dowex-50: analytical grade cation exchange resin - AG- 50W X 4, 200-400 mesh hydrogen form: Bio-Rad Laboratories, Richmond, california U.S.A.

Dowex-1 resin: no special washing necessary. The resin powder can be used directly in the preparation of the columns. Source; Dowex - 1: stock no. 1 x 4-400 chloride form strongly basic anion exchange resin - 4 per cent cross linked - dry mesh 200-400.

Solutions used for Column Chromatography (Appendix V)

All the solutions used throughout the chromatographic procedure, including the de-ionised bi-distilled water, contained 0.1 per cent Triton X-100 unless specified otherwise. The addition of Triton X-100 not only improved the recovery of MOPEG and VMA, but made it also possible to pass effluents and eluates directly over the next column without the formation of air bubbles in its narrow part(208).

Re-pipette dispensers of different volume as indicated below, were used to facilitate delivery of the required volumes of the solutions during the chromatography procedure. As the delivered volume need not be exact, repipette dispensers of the type shown in Fig. 20 (Fisher Scientific Ltd., Toronto, Canada) were used for the purpose. When a number of samples (e.g., 6-8 samples) are analysed simultaneously (as is usually done) a fast delivery becomes essential, making this type of dispenser particularly suitable.

Preparation of the Glass Columns

Glass columns used had an internal diameter of 0.5 cm and were plugged with glass wool (Fig. 21). A short piece of silastic tubing was attached to the bottom end of each column to enable a spring clamp to be used (see below). The colums were mounted on racks in the following manner.

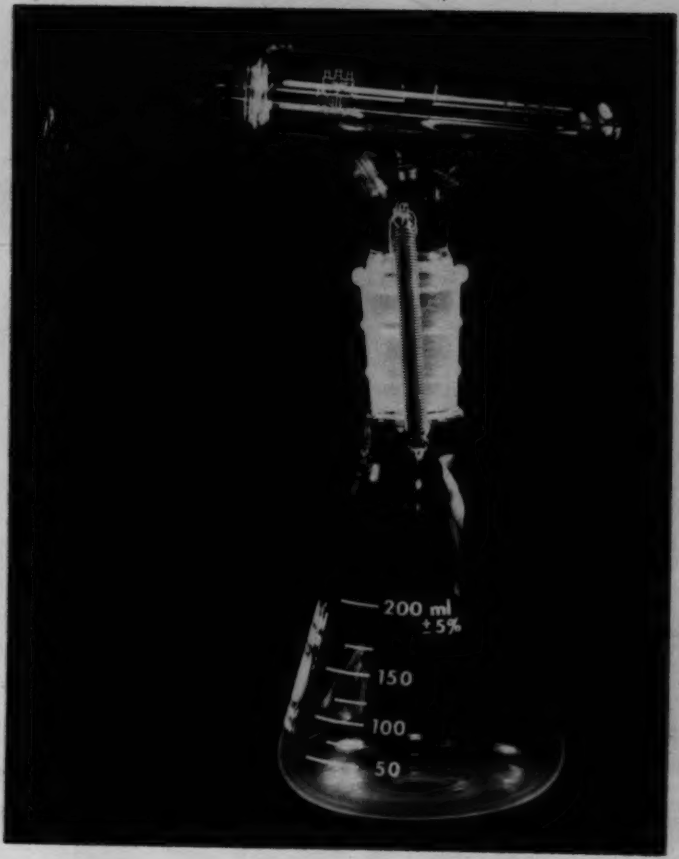

200 ml
±5%
150
100
50

Figure 20. The re-pipette dispensers used for delivery of solutions into the glass columns in the column chromatographic analysis.

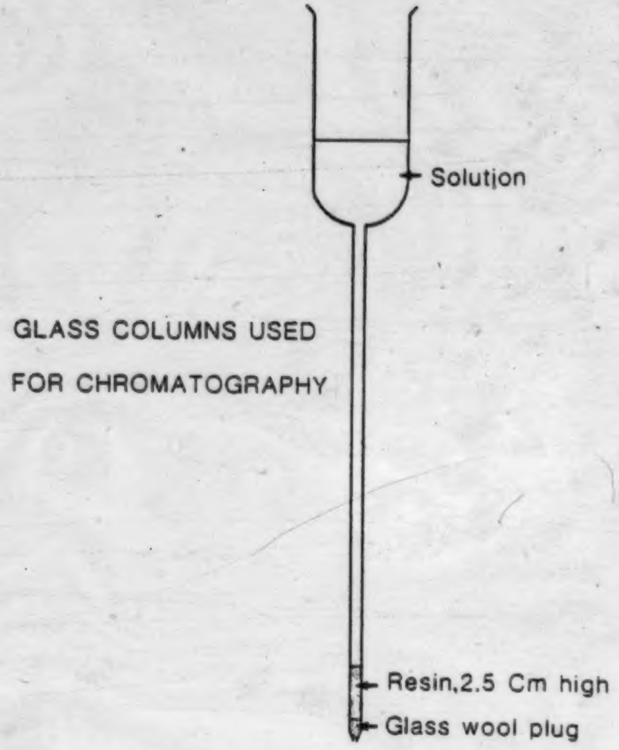

Solution
GLASS COLUMNS USED
FOR CHROMATOGRAPHY
Resin,2.5 Cm high
Glass wool plug

Figure 21. A diagrammatic representation of the glass columns used for the column chromatographic analysis. Internal diameter:0.5 cm.

One three tier rack:

Upper: Alumina columns

Middle: Dowex-50 columns

Lower: Dowex-1 columns

The columns were mounted so that the effluent from the alumina column dropped directly into the Dowex-50 resin column, and the effluent from the latter, directly into the Dowex-1 column.

One two tier rack

Upper: Alumina columns (the glass columns from the top tier of the three tier rack are transferred here).

Lower: Dowex-50 columns

Three single tier racks

Glass columns from the three tier and two tier racks are ultimately transferred to these racks.

Graduated cylinders (with glass stoppers, to facilitate the mixing of the solutions contained within) were labelled A,B,C,D,E, and F for collection of the effluents and eluates, containing intact noradrenaline and its five metabolites. They were numbered 1A, 2A, 3A, 8A depending on the number of samples analysed simultaneously. Twenty-five ml cylinders were adequate for all except the A series where fifty ml cylinders were used.

Dowex-50 columns - Preparation

1. A suspension was prepared by adding one volume (approximately) of distilled water/0.1 per cent Triton X-100 to one volume of the washed Dowex-50 powder in a beaker and stirring continuously with the aid of a magnetic stirrer.

2. 1.0 ml of the Dowex-50 resin suspension was added (while stirring) to each Dowex-50 column. The resin settled at the bottom of the column above the glass wool. The dowex-50 resin should form a column about 2.5 cm in height: if the height was not adequate some more Dowex-50 suspension was added. The beaker containing the unused resin suspension was covered with parafilm and stored in the refrigerator at 4°C for use over the next few days.
3. The following were added to each column

15 ml 2N HCl
5 ml distilled water/0.1 per cent Triton X-100
15 ml sodium phosphate buffer
5 ml distilled water/0.1 per cent Triton X-100

Each solution was added only after the previous solution had drained through the column, i.e, when the meniscus just reached the top of the Dowex-50 resin. Once all four solutions had drained through, the silastic tubing attached to the bottom end of the column was clamped until the experiment proper was begun. It was ensured that a small amount of fluid remained above the Dowex-50 resin to avoid drying of the compound.

4. Dowex-50 columns were placed in the middle tier of the three-tier rack and the lower tier of the two-tier rack.

Dowex-1 columns - Preparation

1. A suspension was prepared by adding one volume of distilled water/0.1 per cent. Triton X-100 to one volume (approximately) of Dowex-1 resin powder in a beaker and stirring with the aid of a magnetic stirrer.

2. 1.0 ml of the resin suspension was added to each Dowex-1 column while stirring. More resin was added, if necessary, to form a Dowex-1 plug approximately 2.5 cm tall. The balance of resin left was kept at 4°C after covering the beaker with parafilm for use over the next few days.
3. 5 ml of 0.1 per cent Triton X-100 was added and the silastic tubing at the bottom clamped once this had drained through the column.
4. The Dowex-1 columns were placed on the lower tier of the three tier-rack.

Column-chromatography - separation procedure

The samples used for the column chromatography were the triads of test tubes containing the superfusate representative of each period of the superfusion experiment. The superfusate collected in the 3 tubes of each triad were pooled together (after removing 1.0 ml from each to count for total radioactivity) and stored immediately at -23°C until the column chromatographic analysis was done. These tubes contained unlabelled NE, DOPEG, VMA, NMN, MOPEG and DOMA to minimise adsorption of the tritiated compound on to the glass. They also had disodium ethylene diamine tetra acetic acid, sodium metabisulphite and hydrochloric acid as protective agents to prevent oxidation of the tritiated compounds (refer Protocol 2.1 for details).

1. While preparing Dowex-50 and Dowex-1 columns the frozen samples were removed from the freezer and allowed to thaw.
2. The contents of each sample tube were mixed with the aid of a vortex stirrer and allowed to settle for 5 minutes. Then 1.0 ml

was pipetted out in duplicate for determination of total radioactivity.

3. Then 10 ml of each sample was pipetted into plastic beakers numbered according to the samples.
4. Approximately 500 mg of activated alumina was added to each beaker containing 10 ml of the samples.
5. A suspension (alumina + sample in beaker) was prepared by continuous stirring with a stirring rod and each sample was titrated to a pH of 8.4. This was done by using 0.5 N Na_2CO_3 for coarse titration and 0.1 N Na_2CO_3 for fine adjustment of the pH. The sample was maintained at pH 8.4 for five minutes using 0.1 N Na_2CO_3 if necessary, while stirring continuously. Note: Na_2CO_3 solutions did not contain Triton X-100.
6. 50 ml graduated cylinders labelled 1A, 2A etc., were placed under the Dowex-1 glass columns in the lower tier of three-tier rack and the titrated samples and the alumina (in the plastic beakers) were added to the glass columns in the upper tier. The plastic beakers were rinsed with distilled water/0.1 per cent Triton X-100 using a squirt bottle, to remove any adherent alumina from the beakers. The clamps attached to the Dowex-50 and Dowex-1 columns of the three-tier rack were removed as the solution drained into these columns from the ones above.
7. Once almost all of the sample had run through the alumina column 5 ml of distilled water/0.1 per cent Triton X-100 was added to remove any alumina adhering to the glass on the upper part of the column. All the solutions added to the alumina columns drained through to the Dowex-50 columns in the middle tier of the three-tier rack. This in turn drained through to the Dowex-1 columns.

DOPEG

8. Once the 15 ml distilled water/0.1 per cent Triton x-100 too had drained through, the alumina columns were removed from the upper tier of the three-tier rack and positioned over the Dowex-50 columns in the two-tier rack (thus the same alumina columns now formed the upper tier of the two tier rack). 25 ml graduated cylinders labelled 1E, 2E, etc. were placed below the Dowex-50 columns in the lower tier of the two-tier rack.

9. The following were added to each column.

   4 x 2 ml 0.2N acetic acid

   Note: added in four stages, 2 ml at a time once the previous 2 ml had run through the column.

10. Alumina columns (upper tier of two-tier rack) were then removed and placed over 25 ml graduated cylinders labelled 1D, 2D, etc., in a single tier rack (refer - DOMA, Step 16).

11. The Dowex-50 columns (the lower tier of the two-tier rack) were rinsed with 5 ml of distilled water/0.1 per cent Triton X-100. The effluent contained DOPEG(E).

NMN

12. Once all the solutions had drained through the Dowex-50 columns in the middle tier of the three-tier rack, (refer Step 7), 5 ml of distilled water/0.1 per cent Triton X-100 was added and allowed to run through.

13. These Dowex-50 columns were removed and placed over 25 ml graduated cylinders labelled 1C, 2C, etc. on a single-tier rack.

14. NMN was eluted with 10 ml of 6N HCL/ethanol solution and collected in the graduated cylinders(C).

DOMA

15. Alumina columns from the upper tier of the two tier rack were positioned over 25 ml graduated cylinders labelled 1D, 2D, etc. (refer steps 10,11).

16. The following solutions were added to elute DOMA:10 ml 0.2 N hydrochloric acid followed by 5 ml 1 N hydrochloric acid once the former had drained through. The solution contained in the graduated cylinders (D) contained DOMA.

NE

17. The Dowex-50 columns remaining in the lower tier of two-tier rack (refer step 12) were removed and positioned over 25 ml graduated cylinders labelled 1F, 2F, etc.

18. NA was eluted from the Dowex-50 by adding 10 ml of 2N hydrochloric acid and collected in the graduated cylinders (F).

MOPEG/VMA

19. Once all the solutions had drained through the Dowex-1 columns in the lower tier of the three-tier rack (refer Step 7) the columns were rinsed with 5 ml of distilled water/0.1 per cent Triton X-100. The effluent was collected in the 50 ml graduated cylinders labelled 1A, 2A, etc. This contained MOPEG.

20. The 50 ml graduated cylinders (A) under the Dowex-1 columns were replaced with 25 ml graduated cylinders labelled 1B, 2B, etc. VMA was eluted with 2 x 10 ml of 2N hydrochloric acid.

Precautions and aids during the procedure:

a) Each solution was applied to the columns only after the previous one had run through the column.

b) The resins were not allowed to dry at any time during the entire procedure. This could be considered as the most important precautionary measure during the procedure. Thus, the menisci in all the columns were observed continuously. However, one need not be frantically worried about this as a 1-2 minute delay from the time the applied fluid reaches the compound to the addition of the next solution does not cause a detectable difference in the radioactivity.

c) A 10 ml syringe with a thin silastic tube attached to its end, was kept handy. This can be helpful when an improper fluid has been applied as one can suck out the wrong fluid immediately, in hope that the assay has not already been ruined.

d) If one applied the strong acid first in Step 17 and then the weak acid, no obvious problem with the counts occurred.

e) A 50 ml cylinder connected to a large bore needle with the needle piercing a rubber stopper that fits tightly at the top rim of the glass columns was kept handy. This was used to apply slight pressure above a fluid contained within a glass column if the drainage was extremely slow.

f) To improve separation of the catechol compounds that are absorbed on to the alumina in step 6 (if the separation is not satisfactory) the following procedure can be adapted. Collect the effluent from the alumina columns (upper tier of three-tier rack) in step 6, following the addition of the superfusate samples, in 25 ml graduated cylinders. Thus, the effluent is not allowed to run into the Dowex-50 and Dowex-1 columns in the middle and lower tiers of the three tier racks (This part of the experiment can be done in a single-tier rack). The effluent is then re-applied to the alumina column and the effluent allowed to run into the Dowex-50 and Dowex-1 columns the second time.

Radioactivity measurements

The volume contained in each of the graduated cylinders was measured and recorded at the end of each stage. The contents of each cylinder was then mixed well and 1.0 ml pipetted out in duplicate and the radioactivity measured in a scintilation counter (refer Superfusion Experiments for details). Each sample was counted for 10 minutes or until 10,000 counts accumulated. Unlike in the Superfusion Experiments the efficiency varied from sample to sample because of different degrees of quenching by the different solutions used in the chromatographic analysis. The efficiency varied from 30-38%. Thus, disintergrations per minute (dpm) were utilised in all calculations involved. As each sample was counted in duplicate for radioactivity, the average dpm contained in 1.0 ml of the sample was first calculated. From this value the amount of radioactivity present in the 16.5 ml (3 x 5.5 ml) of superfusate present in each of the triads of test tubes used for the column chromatographic analysis was calculated (refer Superfusion Experiments for details).

e.g. Volume of superfusate present in triad=16.5 ml

Volume of superfusate used for column chromatography procedure (refer step 3) = 10.0 ml.

a) If the final volume collected in the graduated cylinder A (containing MOPEG) is 44.1 ml, 1.0 ml from this was counted in duplicate for radioactivity. If the mean radioactivity present in 1.0 ml was 91.1 dpm the amount of radioactivity present as

MOPEG in 16.5 ml of the superfusate $= 91.1 \times 44.1 \times \frac{16.5}{10.0}$

$= 6629$ dpm

b) If the total radioactivity present in 1.0 ml of the superfusate (refer to column chromatography procedure, step 2) is 1801.1. The total radioactivity present in 15.0 ml of the superfusate

$= 1801.1 \times 16.5$

$= 29718$

Therefore fraction present as MOPEG

$= 6629/29718$

$= 0.223$

$= 22.3$ per cent

The forms used for the recording of results during column chromatography are shown in Table 1 and Table 2.

```
DATE OF CHROMATOGRAPHIC ANALYSIS              : 13 Oct 1982
DATE OF SUPERFUSION EXPERIMENT                : 11 Oct 1982
TRIAD NO. IN SUPERFUSION EXPERIMENT           : 5
TRIAD CHARACTERISTICS                         :
COLUMN NUMBER                                 : 3
VOLUME OF SAMPLE USED FOR CHROMATOGRAPHY: 10.0ml

     ======================================================================
     :                        :  TOTAL    :   VOLUME      :   VIAL   :
     :                        : VOLUME,ml : COUNTED,ml    :  NUMBER  :
     :=====================================================================
     : ORIGINAL SAMPLE        :    _      :    1.0        :    1     :
     :(TOTAL RADIOACTIVITY)   :           :    1.0        :    2     :
     :---------------------------------------------------------------------
3A   : DOWEX 1 EFFLUENT       :   44.1    :    1.0        :    3     :
     :       (MOPEG)          :           :    1.0        :    4     :
     :---------------------------------------------------------------------
3B   : DOWEX 1 ELUATE         :   14.8    :    1.0        :    5     :
     :       (VMA)            :           :    1.0        :    6     :
     :---------------------------------------------------------------------
3C   : DOWEX 50 ELUATE        :    9.5    :    1.0        :    7     :
     :       (NMN)            :           :    1.0        :    8     :
     :---------------------------------------------------------------------
3D   : ALUMINA ELUATE         :   14.5    :    1.0        :    9     :
     :       (DOMA)           :           :    1.0        :   10     :
     :---------------------------------------------------------------------
3E   : DOWEX 50 EFFLUENT      :   13.7    :    1.0        :   11     :
     :       (DOPEG)          :           :    1.0        :   12     :
     :---------------------------------------------------------------------
3F   : DOWEX 50 ELUATE        :   16.3    :    1.0        :   13     :
     :       (NA)             :           :    1.0        :   14     :
     ======================================================================
```

Table 1: The form used for recording of data during a column chromatography experiment.

DATE OF CHROMATOGRAPHIC ANALYSIS : 13 Oct 1982
DATE OF SUPERFUSION EXPERIMENT : 11 Oct 1982
TRIAD NO. IN SUPERFUSION EXPERIMENT : 5
TRIAD CHARACTERISTICS :
COLUMN NUMBER : 3

METABOLITE FRACTION	VIAL NO.	CPM	EFFICIENCY %	DPM	MEAN DPM	TOTAL VOLUME ml	TOTAL DPM/ 16.5ml	%
TOTAL RADIO -ACTIVITY	1				1801.1	16.5	29718	100
	2							
MOPEG	3				91.1	44.1	6629	22.3
	4							
VMA	5							
	6							
NMN	7							
	8							
DOMA	9							
	10							
DOPEG	11							
	12							
NA	13							
	14							

Table 2: The form used for recording of results during a column chromatography experiment.

Relaxation Experiments:Protocol Three

The results of the Interaction Experiments described above indicated that background TNS inhibited the contraction caused by exogenous noradrenaline in the isolated canine saphenous vein. One possible explanation for this finding is a concurrent relaxatory response produced by the transmural nerve stimulation. The present investigation was an attempt to demonstrate the existence of such a relaxatory response in the canine saphenous vein in vitro, following blockade of the contractile response to TNS.

Protocol 3.1

Lateral saphenous veins were excised from anaesthetised dogs as explained under General Methods. Excess connective tissue was removed and the specimens cut into rings approximately 4 mm in width. Extreme care was taken during the excision and preparation of rings, to avoid contact of surgical instruments with the luminal surface of the blood vessel or rubbing of the "opposing luminal surfaces" against each other, in order to preserve the endothelium. In other rings the endothelium was mechanically removed by inserting the tip of a small tissue forceps into the lumen of the ring and gently rolling the specimen back and forth over a filter paper for 15 seconds(211). The rings were suspended with the aid of two triangular stainless steel clips for isometric tension recording as explained before.

To eliminate the contractile effects of TNS in the venous rings, a combination of guanethidine and phenoxybenzamine was applied to the tissue bath in the following manner. An attempt was made to block the contractile effects of TNS up to a frequency of 32 Hz. As neither guanethidine nor phenoxybenzamine by themselves could not block the contractile effects of TNS adequately, a combination of the two drugs was used. First, guanethidine (10^{-4} mol/l) was introduced into the bath, after the rings were set up as described above, and was present in the bath throughout the experiment. After incubation with guanethidine for 90 minutes, phenoxybenzamine (2×10^{-5} mol/l) was added to the bath and the incubation continued for a further 30 minutes. Then the unbound phenoxybenzamine present in the bath was removed by repeated changes of bath fluid with fresh Krebs solution. During the last 30 minutes of this incubation both propranolol (2×10^{-6} mol/l) and atropine (5×10^{-6} mol/l) were also present in the tissue bath. Thus, they were added to the bath together with the phenxybenzamine at the end of the first 90 minutes of incubation with guanethidine. Atropine and propranolol were added to the bath fluid to prevent any possible muscarinic or beta-receptor mediated relaxation during the subsequent stages of the protocol. These two drugs (and the guanethidine) were present in the bath fluid throughout the rest of the experiment. In order to ensure that the concentration of propranolol used (2×10^{-6} mol/l) was sufficient to prevent beta-receptor mediated relaxation, the response to isoprenaline was tested in some rings. Isoprenaline in a concentration of 2×10^{-5} mol/l did not produce any relaxation in saphenous vein rings pre-contracted with prostaglandin $F_{2\alpha}$ as described below. The concentration of atropine used was similar to that used by other workers to block any possible muscarinic relaxation in smooth muscle(212).

At the end of this period of two hours, the preparations were contracted by adding Krebs buffer solution containing prostaglandin $F_{2\alpha}$ (10^{-5} mol/l). The contraction produced was approximately 80 per cent of the maximum for prostaglandin $F_{2\alpha}$ in the saphenous vein.

Once this contraction reached a plateau, the response to TNS was determined using trains of stimuli of 30 second duration at each frequency. The stimulus parameters used were as follows: square wave pulses; duration 1.0 ms, strength 10 V, frequency 1,2,4,8,16, and 32 Hz. Each train of pulses was applied following complete recovery from the response to the previous train of stimuli. This period varied from 8-30 minutes.

Protocol 3.2

In Protocol 3.1, TNS applied after pre-contraction of the saphenous vein with prostaglandin $F_{2\alpha}$ produced a frequency dependent relaxation. Protocol 3.2 was done to elucidate further, the mechanism responsible for this relaxatory response to TNS. Protocol 3.1 was carried out first to determine the stimulus-response curve to TNS, as a control. Then one of the following drugs was added to the tissue bath (after replacing the bath fluid with fresh buffer containing prostaglandin $F_{2\alpha}$) and the stimulus-response curve repeated after 30 mintues of incubation with the drug.

1. Fast Na^+ channel inhibitor tetrodotoxin (10^{-6} mol/l)
2. H_2-receptor antagonist cimetidine (10^{-4} mol/l)
3. Cyclo-oxygenase inhibitor indomethacin (10^{-5} mol/l)
4. P_1-receptor antagonist aminophylline (10^{-5} mol/l)
5. Na^+/K^+ adenosine triphosphatase inhibitor ouabain (10^{-4} mol/l)
6. Free radical scavengers ascorbic acid (10^{-4} mol/l) or catalase (50 µg/ml)
7. The stimulus-response curves were also repeated following incubation of the ring in zero-K^+ "Krebs-bicarbonate" solution containing prostaglandin $F_{2\alpha}$ for 30 minutes. Zero-K^+ "Krebs-

bicarbonate" solution was prepared by replacing the KCl in the Krebs-bicarbonate buffer solution used, with an equimolar concentration of NaCl.

8. Only one drug was tested in each ring. Control rings without the above drugs were run in parallel to correct for changes in the responses with time. The stimulus-response curves to TNS before and after each drug were compared by regression analysis after log transformation (to base 2.0) of the frequency values.

Protocol 3.3

As tetrodotoxin used in Protocol 3.2 did not abolish the relaxatory response to TNS, the neural origin of this response was investigated using cold storage of the saphenous veins. Intramural autonomic nerves are known to degenerate when excised blood vessels are stored at 4°C(213). Some excised saphenous veins were stored in Krebs buffer solution at 4°C for 9 days as described by Guimaraes et al(214). Rings were mounted in the tissue bath following this cold storage and the response to intermittent trains of TNS determined after pre-contraction with prostaglandin $F_{2\alpha}$ as described in Protocol 3.1. The stimulus-response cruves to TNS in these cold-stored veins were compared with the stimulus-response curves done on rings prepared from the same veins on the day of excision. Although the contractile response to TNS was abolished in these cold stored veins, guanethidine and phenoxybenzamine were applied to some of these rings to simulate the conditions in the control rings. Comparisons were made using a regression analysis on the stimulus-response curves.

Protocol 3.4

The effect of chemical sympathetic denervation using 6-

hydroxydopamine on the relaxatory response to TNS was investigated here. The method used for the denervation was a modification (personal communication Vanhoutte PM, Lorenz RR) of the method used by Aprigliano and Hermsmeyer(215). The rings were set up for isometric tension recording as before. Aeration of the tissue bath was stopped and 20 ml of unbuffered physiological salt solution (for electrolyte composition refer Appendix III) containing 6.0 mg of dissolved 6-hydroxydopamine (1.46×10^{-3} mol/l) was added to the tissue bath in place of Krebs buffer solution. Incubation with 6-hydroxydopamine was carried out for 10 minutes. The bath solution was replaced with another 20 ml of physiological salt solution containing 6-hydroxydopamine and the incubation continued for a further 10 minutes. The 6-hydroxydopamine was then removed and replaced with Krebs buffer solution and aeration with 95 per cent O_2-5 per cent CO_2 recommenced. Frequent rinsing with fresh Krebs solution was carried out over the next 3 hours until the tension (increased by the 6-hydroxydopamine treatment) returned to the control level. The rings were then contracted with prostaglandin $F_{2\alpha}$ (in the presence of propranolol and atropine as before) and the stimulus-response curve to TNS repeated. In some of these 6-hydroxydopamine treated veins, guanethidine and phenoxybenzamine were also applied to simulate the conditions in Protocol 3.1. In Protocol 3.3 rings without 6-hydroxydopamine treatment were run in parallel as controls. The stimulus-response curves in the 6-hydroxydopamine treated rings were compared with the stimulus-response curves in the control rings.

Protocol Four

As a background contraction produced by exogenous noradrenaline appeared to enhance the response to TNS in Protocol 1.2, it was decided to investigate this phenomenon further. The effect of low concentrations of exogenous noradrenaline (which by itself did not produce any increase in tension in the saphenous vein), on the contractile response to TNS was investigated here. Saphenous vein rings were prepared for experimentation as explained in the General Methods. After an equilibration period of 90 minutes TNS was applied as 5 second trains of stimuli at 8 Hz (strength:10V, pulse duration:0.3 ms) every 5 minutes until the contractile response to the train of TNS became stable. This stable value was regarded as the control and the stimulus parameters were maintained constant for the rest of the experiment. Next, exogenous noradrenaline was added to the tissue bath in a concentration of -9.0 $\log_{10}$ mol/l and the response to the trains of TNS determined. After three to four trains of TNS the exogenous noradrenaline was removed by several changes of the bath fluid, while continuing the TNS every 5 minutes. Once the contractile response to TNS reached the control value the experiment was repeated with a higher concentration of exogenous noradrenaline. The concentrations of exogenous noradrenaline utilised for the protocol were as follows (in $\log_{10}$ mol/l): -9.0, -8.5,-8.0, -7.5, -7.2.

The maximum contractile response to TNS in the presence of each concentration of exogenous noradrenaline was expressed as a percentage of the control. The experimental values at each concentration of background exogenous noradrenaline and the control values were analysed as a two-way analysis of variance (the control and the responses to TNS at the five concentrations of noradrenaline constituting 6 treatment groups). When the F value was significant ($p<0.05$), the differences between any two treatment groups were assessed with the least significant difference test.

STATISTICAL ANALYSIS(216,217)

For each Protocol the number of experiments reported corresponds to the number of dogs used unless specified otherwise (see Protocol 1.1 and Protocol 1.2). All data are expressed as mean ± standard error of mean (SEM). For statistical comparison of two sets of observations, the Student's t-test (paired or unpaired as appropriate) was employed; a p value of less than 0.05 was considered as a statistically significant difference between two groups of data.

The control and experimental stimulus-response curves to TNS in Protocol Three were compared as follows: A regression analysis (after log transformation to base 2.0 of the frequency values) was carried out first on each group (experimental and control) of data. The two regression lines resulting from the above were compared thereafter. The slopes of the two lines were compared initially, followed by the elevation of the lines above the x-axis (i.e, homogeneity of the Y-intercept) if the slopes were found not to differ significantly. The latter procedure was carried out by an analysis of covariance.

The control and the experimental values in Protocol Four were analysed with a two-way analysis of variance, taking each day's experiment as a single replicate. The control response to TNS and the responses at the 5 different concentrations of exogenous noradrenaline were regarded as 6 treatment groups. When the F value was significant the differences between any two treatment groups were assessed with the least significant difference test.

RESULTS

Protcol One

Saphenous vein strips mounted for isometric tension recording, in the tissue bath, did not demonstrate any spontaneous mechanical activity. They responded to exogenous noradrenaline in a concentration dependent manner. With repeated changing of bath fluid the tension returned to approximately the same level as before the commencement of the dose-response curve. This recovery from a dose-response curve took from 30-45 minutes. TNS applied as square-wave pulses produced a frequency dependent contraction with a maximum contraction at 16-32 Hz. The maximum contraction produced by TNS in the saphenous vein strips was about 90 per cent of the maximum contraction produced by exogenous noradrenaline. Here too the tension returned to control levels following cessation of stimulation. Although this occurred even without washing, several changes of bath fluid were done during this period which varied from 15-20 minutes. Further, the bath fluid was changed every 30 minutes even when no specific intervention was carried out. In 22 preparations (strips) the basal tension was 3.2 g (mean ± 0.18 g SEM) and the maximum tension to exogenous noradrenaline was 4.9 g (mean ± 0.30 g SEM).

Protocol 1.1

The effect of a TNS induced background contraction, on the contraction produced by exogenous noradrenaline was examined here. The experiment was carried out using different concentrations of exogenous noradrenaline and different magnitudes of background contraction produced by TNS as explained under Methods. The concentrations of exogenous noradrenaline used ranged from 7.5×10^{-7} mol/l to 6.0×10^{-6}

mol/l. The magnitude of the control contractions produced by this exogenous noradrenaline alone in Step C and Step E of the protocol ranged from 30-90 per cent of the maximum contraction for noradrenaline. The background contractions produced by TNS at different frequencies ranged from 5-80 per cent of the maximum contraction for exogenous noradrenaline in the saphenous vein. The additional contraction produced by exogenous noradrenaline, when added against a background contraction induced by TNS in Step D was found to be much less than the controls, i.e., Step C and Step E. Often the total contraction produced by TNS and exogenous noradrenaline in Step D was only slightly greater than the contraction produced by exogenous noradrenaline alone. On some occasions they were equal. An example from Protocol 1.1 is shown in Figure 22.

As explained under Methods, a direct comparison between the additional contraction produced by exogenous noradrenaline against a background of TNS, and the contractions produced by exogenous noradrenaline alone would be erroneous. Thus, an expected contraction was calculated for each concentration of exogenous noradrenaline used in Protocol 1.1, depending on the magnitude of the background contraction produced by TNS (see Methods). Following this correction, the observed contractions were compared with the expected contractions. The results are summarised in Fig. 23. Seventy-eight observations were made on 12 saphenous veins (number of dogs = 12). The observed contractions in Step D were significantly less than the expected contractions with the observed/expected ratio having a mean of 82.4 per cent (SEM 2.1 %, $p<0.001$). The individual data points utilised for Figure 23 are shown in Figure 24, where the observed/expected contraction ratios were

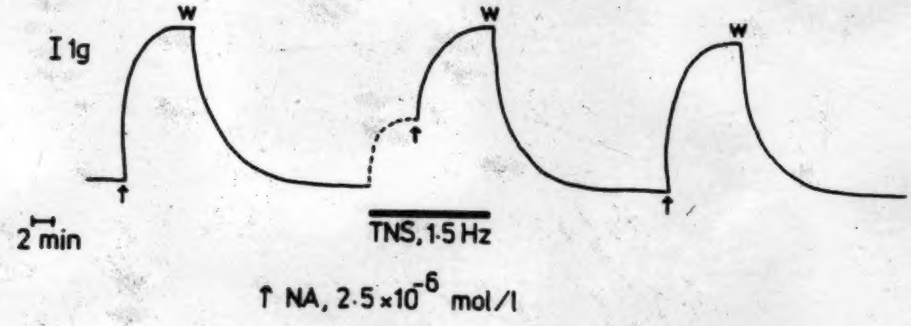


Figure 22. An example from Protocol 1.1. The additional contraction produced by exogenous noradrenaline (NA) added against a background contraction by transmural nerve stimulation (TNS), is much less than the controls (on either side); in fact, the total contraction produced by exogenous noradrenaline and TNS in the middle is not much different from the contraction produced by exogenous noradrenaline alone in the two controls.

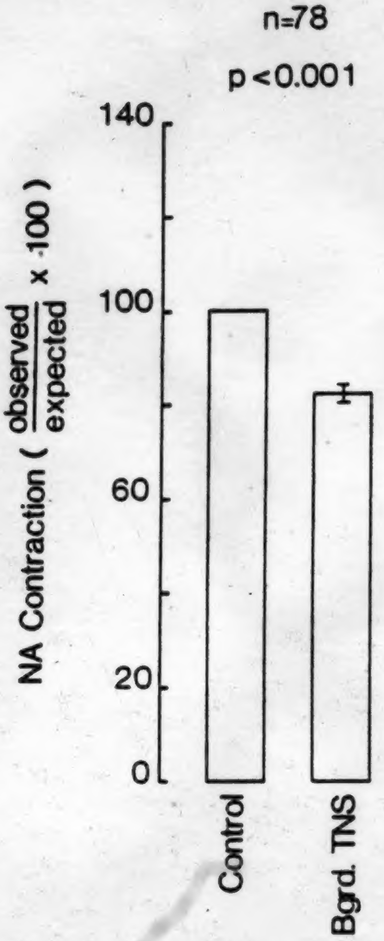


Figure 23: The summary of the results from Protocol 1.1. The observed/expected contraction produced by exogenous noradrenaline (NA) added against a background contraction produced by transmural nerve stimulation (Bgrd. TNS) is significantly less than the controls.

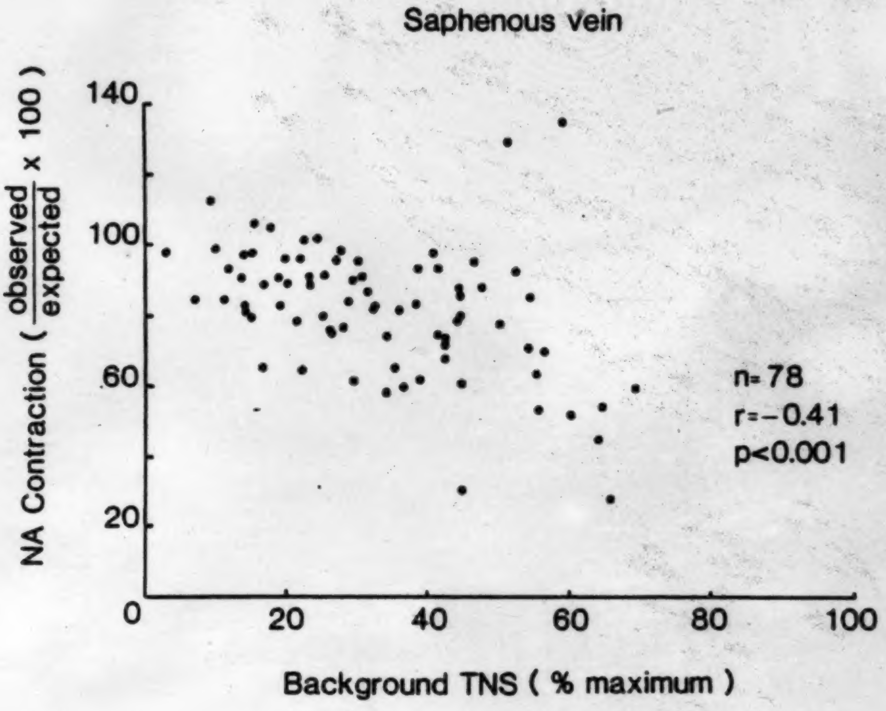


Figure 24. The individual data points for the observed/expected contractions by exogenous noroadrenaline (NA) added against a background contraction produced by transmural nerve stimulation (TNS) in Protocol 1.1, plotted against the magnitude of the background TNS contraction. The latter is expressed as a percentage of the maximum contraction produced by exogenous nroadrenaline in the canine saphenous vein.

plotted against the magnitude of the background contraction ratios produced by TNS. The latter was expressed as a percentage of the maximum contraction for exogenous noradrenaline. As can be seen, 71 of 78 data points for observed contractions were less than the expected contractions. Further, the observed/expected ratio appeared to diminish with increasing magnitude of the background contraction produced by TNS, with a significant negative correlation ($r = -0.42$, $p<0.001$). Correlation coefficient although significant, was rather low with a coefficient of determination (r^2) of 0.17.

Protocol 1.2

The effect of a exogenous noradrenaline induced background contraction, on the contraction produced by TNS was examined here. The experiment was carried out using different frequencies of TNS and different magnitudes of background contraction produced by exogenous noradrenaline as explained under Methods. The control contractions produced by TNS in Step C and Step E of the protocol ranged from 20-80 per cent of the maximum contraction for exogenous noradrenaline in the vein strips (The upper limit was constrained by the fact that maximum contraction to TNS in the saphenous vein was about 90 per cent of the maximum contraction to exogenous noradrenaline). The background contractions produced by exogenous noradrenaline in Step D, ranged from 5-75 per cent of the maximum contraction to exogenous noradrenaline. The additional contraction produced by TNS applied against the background contraction induced by exogenous noradrenaline, although less than the controls, was more than that observed in Step D of Protocol 1.1 (which was the reciprocal of Step D in Protocol 1.2). This additional contraction was occasionally observed to be equal to the control contraction produced by TNS alone. A representative example from Protocol 1.2 is shown in Fig. 25.

As a direct comparison between the additional contractions produced by TNS in Step D with the two control contractions in Step C and Step E was not possible, expected contractions were calculated. The results are summarised in Fig. 26. Seventy one observations were made on 12 saphenous veins (number of dogs = 12). Unlike in Protocol 1.1 where the observed contractions in Step D were significantly less than the expected contractions, the observed contractions were significantly more than the expected contractions in Protocol 1.2 with the observed/expected ratio having a mean of 132.8 per cent (SEM 3.6 %, $p<0.001$). The individual data points utilised for Figure 26 are shown in Figure 27 where the observed/expected contraction ratios were plotted against the magnitude of the background contraction produced by exogenous noradrenaline, the latter being expressed as a percentage of the maximum contraction for exogenous noradrenaline. As can be seen, 64 (of 71) observed contractions were greater than the expected contractions. Further, the data points appeared to be randomly distributed, the observed/expected ratio showing no significant correlation with the magnitude of the background contraction produced by exogenous noradrenaline ($r=+0.06$, $p>0.05$).

Protocol 1.3

This protocol was carried out to determine whether the inhibitory effect of TNS on the exogenous noradrenaline contraction was due to the excitation of intramural nerves or due to the field of electrical current per se.

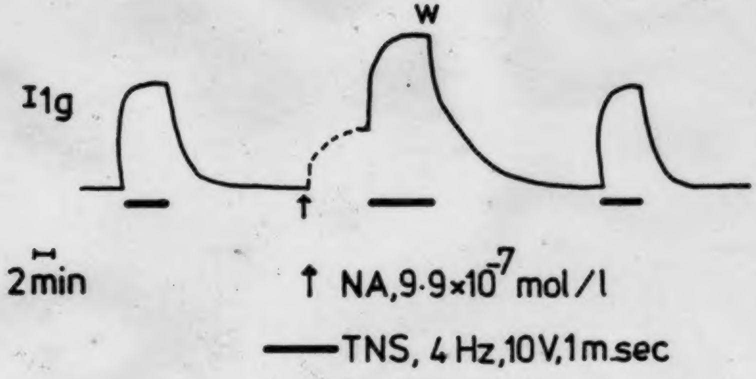


Figure 25. An example from Protocol 1.2. The additional contraction produced by transmural nerve stimulation (TNS) applied against a background contraction by exogenous noradrenaline (NA) although less than the controls (on either side) is relatively more than that observed in Protocol 1.1 (refer Fig. 22) where exogenous NA was added against background TNS, i.e., the reciprocal of Protocol 1.2.

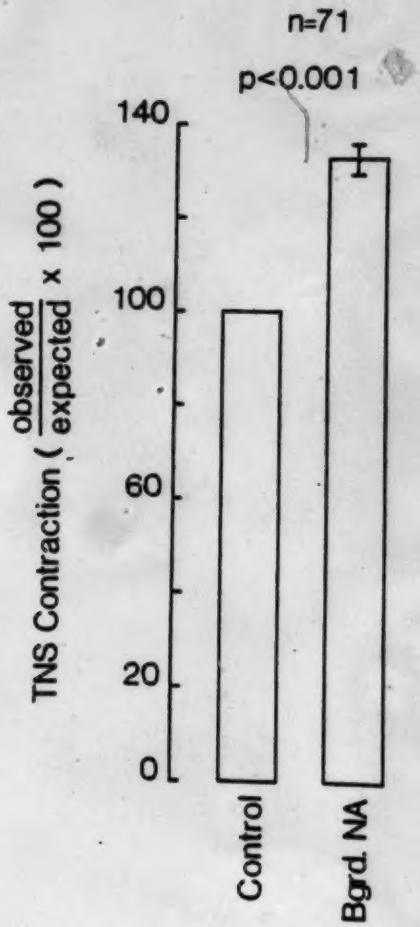


Figure 26. The summary of the results from Protocol 1.2. The observed/expected contractions produced by transmural nerve stimulation (TNS) applied against a background contraction by exogenous noradrenaline (Bgrd. NA) is significantly more than the controls.

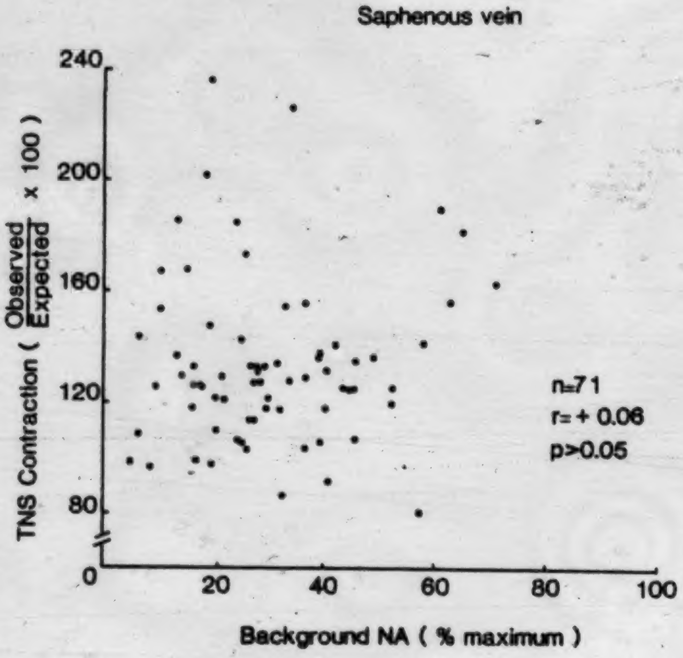


Figure 27. The individual data points for observed/expected contractions produced by transmural nerve stimulation (TNS) applied against a background contraction by exogenous noradrenaline (NA) in Protocol 1.2. The observed/expected contractions are plotted against the magnitude of the background NA contraction. The latter is expressed as a percentage of the maximum contraction produced by exogenous NA in the canine saphenous vein. As can be seen, 64 of the 71 data points for the observed contractions are more than the expected contractions, i.e., more than 100 per cent on the y-axis.

Steps A to E of protocol 1.1 were carried out first. This showed results similar to that observed in Protocol 1.1. Thus, TNS was found to inhibit the contraction produced by exogenous noradrenaline. The contractile response to the frequency of TNS used in Step D was blocked by the addition of guanethidine into the tissue bath. The concentration of guanethidine necessary for the blockade varied from 1 x 10^{-6} mol/l to 5 x 10^{-6} mol/l depending on the frequency of TNS used in Step D. A representative example of Step C, Step D and Step E following this blockade is shown in Fig. 28. The contraction produced by exogenous noradrenaline in Step D was similar to the two controls (Step C, Step E) in spite of the electrical current flowing between the platinum electrodes during Step D. The contraction in Step D was compared with the mean of the two controls. The results are summarised in Fig. 29. The mean contraction produced by exogenous noradrenaline during the passage of an electrical current was 99.8 per cent (mean ± 0.5% SEM) of the controls ($n=8$, $P>0.05$).

A similar experiment was carried out using the calcium antagonist diltiazem hydrochloride in place of guanethidine. The concentration of diltiazem required to block the contractile effects of TNS varied from 10^{-5} to 10^{-4} mol/l depending on the frequency of TNS used in Step D. At these concentrations the contractile response to exogenous noradrenaline was diminished by only 20-30 per cent. As with guanethidine, the contraction produced by exogenous noradrenaline was not modified by the passage of an electrical current through the solution during Step D. The mean contraction produced by exogenous noradrenaline in Step D was 99.9 per cent (mean ± 2.3 per cent SEM) of the controls ($n=9$, $p>0.05$).

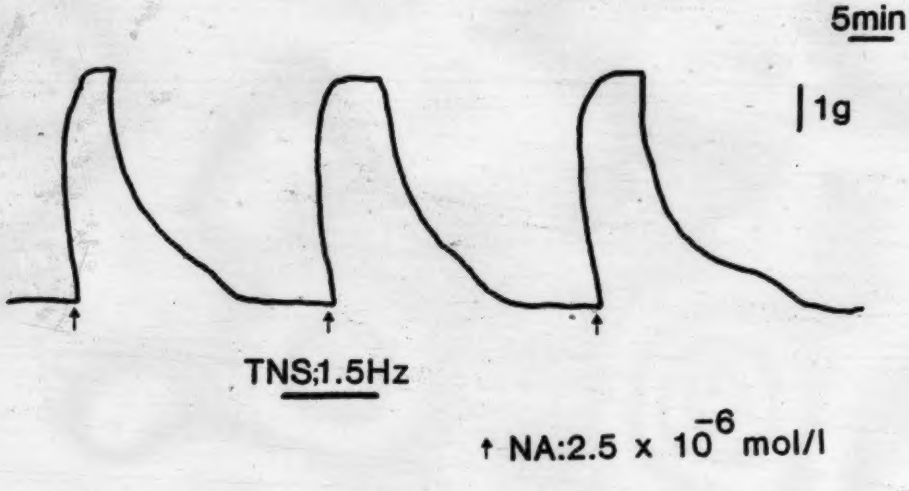


Figure 28. A representative example from Protocol 1.3. The two control contractions to exogenous noradrenaline alone (on either side) and the contraction to the same concentration of noradrenaline added while applying TNS (middle) are shown. All three responses were elicited in the presence of guanethidine in a sufficient concentration to block the contractile effect of TNS at 1.5 Hz.

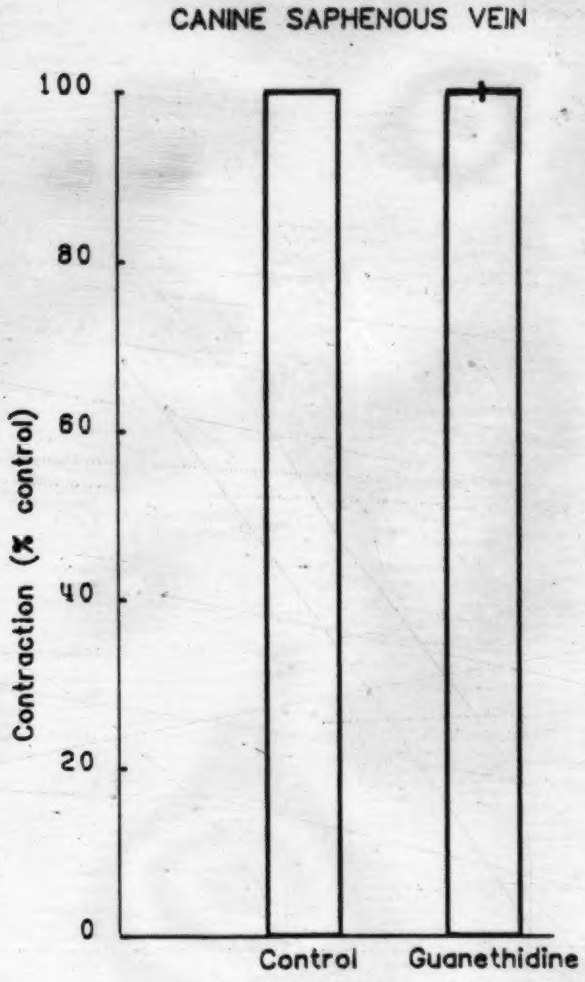


Figure 29. Summary of results from Protocol 1.3. The contraction produced by exogenous noradrenaline (y-axis) in the controls is compared with the contraction produced by the same concentration of noradrenaline added against a background of transmural nerve stimulation (TNS) with the contractile effect of TNS blocked with guanethidine (n=8, p>0.05).

Protocol 1.4

These experiments were designed to determine whether the inhibitory effect of background TNS, on the exogenous noradrenaline mediated contraction, observed in Protocol 1.1 was specific for TNS or whether it would still be evident when another agonist is substituted in place of TNS.

The response to exogenous noradrenaline added against a background contraction produced by TNS was compared with the response to the same concentration of exogenous noradrenaline added against a background contraction produced by tyramine (or methoxamine or histamine or phenylephrine). In this protocol, the additional response to exogenous noradrenaline was expressed as a percentage of the mean of the two controls i.e., Step C and Step E. The background contraction produced by TNS was matched (as close as possible) to that produced by tyramine to enable a direct comparison of the additional responses to be made. All four compounds, tyramine, methoxamine, histamine and phenylephrine produced a concentration dependent contraction in saphenous vein strips/rings. Dose-response curves to each of these drugs were performed in a preliminary set of experiments to get an idea of the concentration range to be used in the protocol proper. Tyramine, methoxamine and phenylephrine produced complete dose-response curves at a concentration range from 10^{-7} to 10^{-4} mol/l. In the case of tyramine, the dose-response curves were repeated in the presence of cocaine (10^{-5} mol/l) to determine the concentration range in which its direct effects (on the smooth muscle) could be avoided. In the presence of cocaine, tyramine produced no appreciable contractions up to a concentration of 6 x 10^{-5} mol/l. In the protocol proper the concentration of tyramine used was kept below this to avoid direct effects on smooth muscle. Saphenous veins were far less responsive to histamine compared with the adrenergic agonists described above. It often produced no contractions until a concentration of 10^{-4} mol/l was reached.

A representative example from the experiments using tyramine hydrochloride is shown in Fig. 30. The additional contraction produced by exogenous noradrenaline against a background contraction induced by tyramine, was much greater than that produced against a background contraction induced by TNS. This difference was apparent with different magnitudes of background contraction as well as different concentrations of exogenous noradrenaline. The pooled results are summarised in Fig. 31 where the additional contraction produced by the exogenous noradrenaline is expressed as a percentage of the controls. The additional contraction against a background of TNS was 67.1 per cent (mean ± 2.5% SEM) of the control, while the additional contraction against a background of tyramine was 86.5 per cent (mean ± 3.4% SEM) of the control ($n=16$, $p<0.001$).

In the experiments using phenylephrine hydrochloride as the background agonist, the drug was used in a concentration range from 1×10^{-6} mol/l to 4×10^{-6} mol/l. The pooled results of these experiments are summarised in Fig. 32. The additional contraction produced by exogenous noradrenaline against a background of TNS was 56.2 per cent (mean ± 3.3%, SEM) of the control while the additional contraction against a background of phenylephrine was 68.3 per cent (mean ± 3.4% SEM) of the control. The additional contraction against a background of phenylephrine was significantly greater than that against a background of TNS ($p<0.001$, $n=11$).

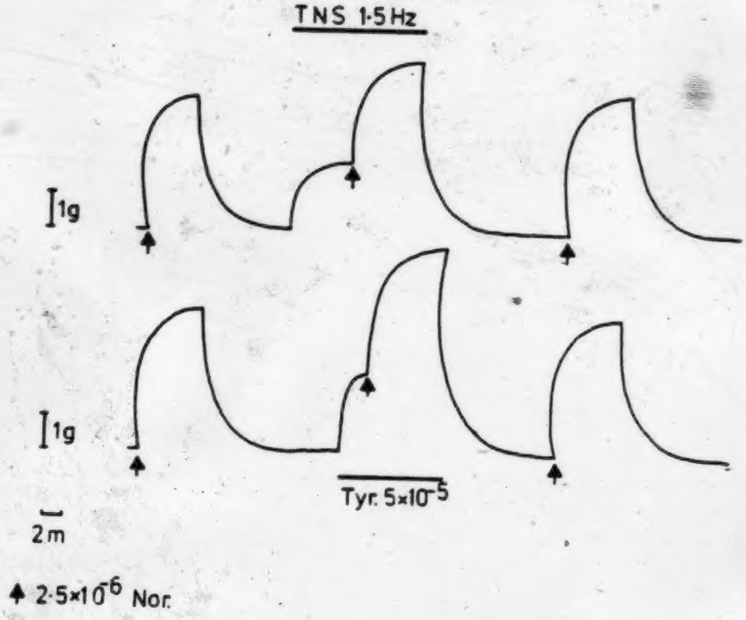


Figure 30. A representative example from Protocol 1.4 with tyramine hydrochloride (Tyr) as the background agonist. The response to exogenous noradrenaline (Nor) added against a background contraction by transmural nerve stimulation (TNS) (Upper middle), the response to the same concentration of noradrenaline added against a background contraction by tyramine (lower middle) and the four control contractions to exogenous noradrenaline alone are shown. The additional contraction produced by exogenous noradrenaline against a background contraction by tyramine is much more than that produced against a background contraction by TNS.

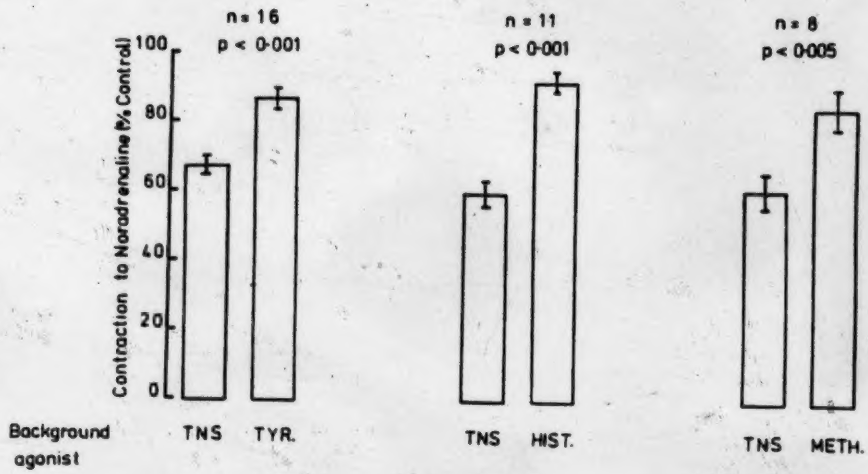


Figure 31. Results from Protocol 1.4 (background agonists:tyramine, histamine and methoxamine). The additional contraction produced by exogenous noradrenaline against a background contraction by transmural nerve stimulation (TNS) is compared with the additional contraction to the same concentration of noradrenaline against a background contraction by tyramine (left), histamine (middle) and methoxamine (right). The additional contraction to noradrenaline is expressed as a percentage of the control. Control refers to the contraction produced by exogenous noradrenaline alone, i.e., with no background agonist or TNS. Tyr: tyramine, Hist:histamine, Meth:methoxamine.

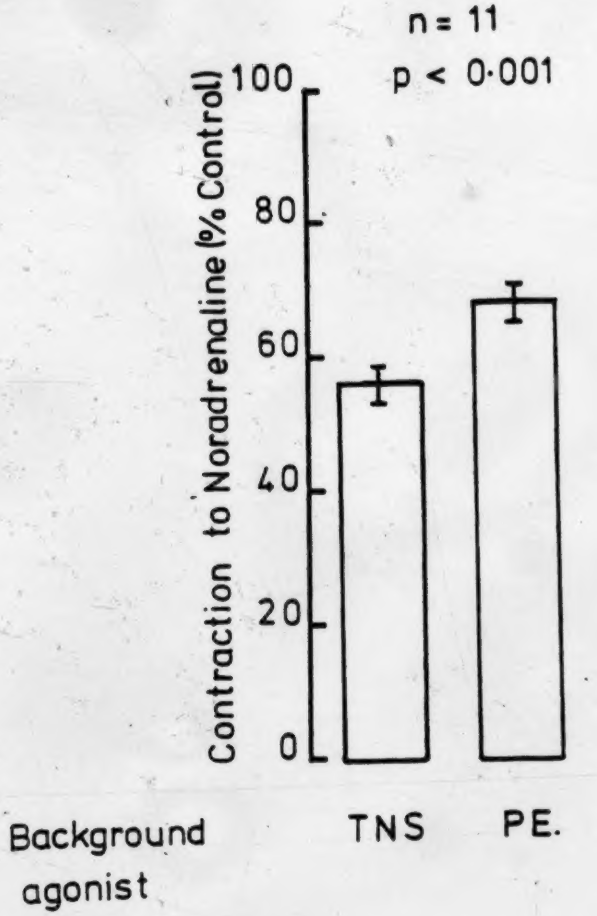


Figure 32. Summary of results from Protocol 1.4 (background agonist: phenylephrine). The additional contraction produced by exogenous noradrenaline against a background contraction by transmural nerve stimulation (TNS) is compared with the additional contraction to the same concentration of noradrenaline against a background contraction by phenylephrine. The additional contraction to noradrenaline is expressed as a percentage of the control. Control refers to the contraction produced by noradrenaline alone, i.e., with no background agonist or TNS. PE: phenylephrine.

In the experiments using methoxamine hydrochloride as the background agonist, the drug was used in a concentration range from 1 x 10^{-6} mol/l to 6 x 10^{-6} mol/l. The pooled results of these experiments are summarised in Fig. 31. The additional contraction produced by exogenous noradrenaline against a background of TNS was 60.8 per cent (mean ± 5.4% SEM) of the control while the additional contraction against a background of methoxamine was 84.3 per cent (mean ± 5.6% SEM) of the control. The additional contraction against a background methoxamine was significantly greater than that against a background of TNS ($p < 0.001$, n=8).

In the experiments using histamine dihydrochloride as the background agonist, the drug was used in a concentration range from 5 x 10^{-5} mol/l to 4 x 10^{-4} mol/l. The pooled results of these experiments are summarised in Fig. 31. The additional contraction produced by exogenous noradrenaline against a background of TNS was 59.6 per cent (mean ± 3.2% SEM) while the additional contraction against a background of histamine was 91.7 per cent (mean ± 2.6% SEM). The additional contraction against a background of histamine was significantly greater than that against a background of TNS ($p < 0.001$, n=12).

Protocol 1.5

This protocol was designed to investigate the mechanism responsible for the inhibition observed in Protocol 1.1, utilising "antagonists" to some possible mediators of the inhibitory phenomenon.

The effect of propranolol, indomethacin, aminophylline and cimetidine on the inhibition of the exogenous noradrenaline mediated contraction by background TNS was investigated here. Thus, Step C, Step D and Step E of Protocol 1.1 were carried out first and then repeated in the presence of one of the above mentioned drugs. The additional contractions produced by exogenous noradrenaline against a background of TNS with and without the drug were compared. Each was expressed as a percentage of the mean of the two controls, i.e, Step C and Step E.

In the experiments using the β blocker propranolol it was used in a concentration of 10^{-5} mol/l. This concentration of propranolol was sufficient to block the relaxant effects of the beta-agonist isoprenaline (following α-blockade) in the canine saphenous vein. This was established in preliminary experiments. At this concentration, the contractile response to exogenous noradrenaline was increased by up to 10 per cent in some preparations. The pooled results from the experiments using propranalol are summarised in Fig. 33. The additional contraction produced by exogenous noradrenaline was 66.3 per cent (mean ± 4.6%, SEM) of the control in the absence of propranolol, and 58.4 per cent (mean ± 3.0% SEM) in the presence of the drug. The latter value was significantly less than the former ($0.05 > p > 0.01$, $n=10$).

In the experiments using indomethacin, the drug was used in a concentration of 10^{-5} mol/l. At this concentration it did not modify appreciably the magnitude of the contractions produced by exogenous noradrenaline or TNS. The pooled results of these experiments are summarised in Fig. 34. The additional contraction produced by exogenous noradrenaline was 67.8 per cent (mean ± 1.9% SEM) of the control in the absence of indomethacin and 65.0 per cent (mean ± 2.2% SEM) of the control in the presence of the drug. These two values were not significantly different from each other ($p > 0.05$, $n=11$).

In the experiments using the P_1-purinoceptor antagonist, aminophylline, the drug was used in a concentration of 10^{-5} mol/l. At

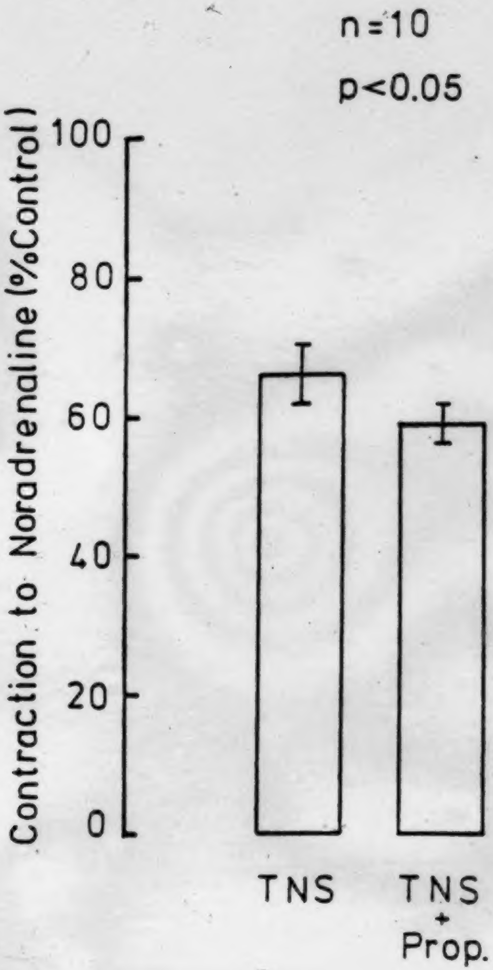


Figure 33. Results from Protocol 1.5 (propranolol). The additional contraction produced by exogenous noradrenaline against a background contraction by transmural nerve stimulation (TNS) with and without propranalol (10^{-5} mol/l) are shown. The additional contraction to noradrenaline is expressed as a percentage of the control. Control refers to the contraction produced by noradrenaline alone, i.e., with no background TNS. Prop:Propranolol.

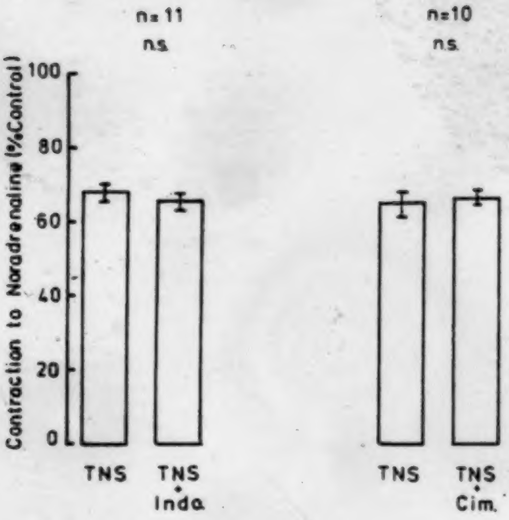


Figure 34. Results from Protocol 1.5 (indomethacin and cimetidine). The additional contraction produced by exogenous noradrenaline against a background contraction by transmural nerve stimulation (TNS) with and without (a) indomethacin 10^{-5} mol/l (left) and (b) cimetidine 10^{-5} mol/l (right) are shown. The additional contraction to noradrenaline is expressed as a percentage of the control. Control refers to the contraction produced by noradrenaline alone, i.e., with no background TNS. Indo:indomethacin, Cim:cimetidine.

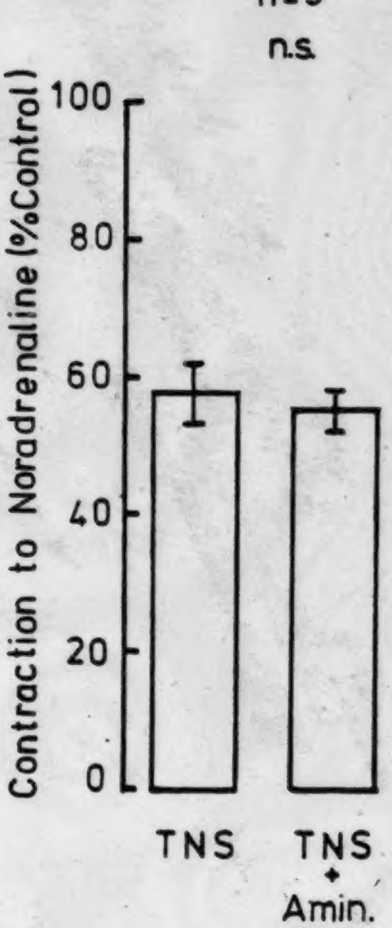


Figure 35. Results from Protocol 1.5 (aminophylline). The additional contraction produced by exogenous noradrenaline against a background contraction by transmural nerve stimulation (TNS) with and without aminophylline (10^{-5} mol/l) are shown. The additional contraction to exogenous noradrenaline is expressed as a percentage of the control. Control refers to the contraction produced by noradrenaline alone, i.e, with no background TNS. Amin:aminophylline.

this concentration aminophylline did not significantly affect the contractile responses to exogenous noradrenaline or TNS. As with indomethacin, the additional contraction produced by exogenous noradrenaline against a background of TNS did not appear to be modified by the presence of aminophylline. The pooled results of these experiments are summarised in Fig. 35. The additional contraction produced by exogenous noradrenaline was 57.8 per cent (mean ± 4.6% SEM) of the control in the absence of aminophylline and 55.4 per cent (mean ± 3.1%, SEM) of the control in the presence of the drug. These two values were not significantly different from each other ($p>0.05$, $n=9$).

In the experiments using cimetidine the drug was used in a concentration of 10^{-5} mol/l. The presence of cimetidine did not cause any appreciable alteration in the magnitude of the contractile responses to TNS or exogenous noradrenaline. The pooled results from these cimetidine experiments are summarised in Fig. 34. The additional contraction produced by exogenous noradrenaline was 65.3 per cent (mean ± 2.6% SEM) of the control, in the absence of cimetidine and 66.4 per cent (mean ± 2.4% SEM) of the control in the presence of the drug. These two values were not significantly different from each other ($p>0.05$, $n=10$).

Protocol Two

This protocol was carried out to determine whether (1) pre-synaptic α_2-inhibition takes place during the experimental conditions utilised in Protocol 1.1 and Protocol 1.2 and (2) if so, whether these pre-synaptic effects could account for the inhibitory phenomenon observed in Protocol 1.1 and the difference in results between Protocol 1.1 and Protocol 1.2.

Protocol 2.1

Step D of Protocol 1.1 was carried out as a superfusion experiment in this part of the study. The radioactivity measurements in the superfusate have been expressed below as fractional release per 2 minute period of collection. All values have been multiplied by 10^3 for the sake of clarity as the fractional release is almost always a number with two to three decimal places. Thus, the fractional release values given below have to be multiplied by 10^{-3} in order to obtain the true fractional release.

Two hours after the commencement of the superfusion, the experiment proper was begun. At this time, the basal efflux of tritiated compounds had reached a steady level. In five experiments the total radioactivity in the superfusate at this time was 1.58 (mean ± 0.10 SEM). Unmetabolised ^{3}H-noradrenaline constituted only 4.3 per cent of this total radioactivity. The deaminated compound DOPEG (3,4-dihydroxyphenylglycol) formed 34.3 per cent of this fraction and the ortho-methylated deaminated compound MOPEG (3-methoxy, 4-hydroxyphenylglycol) 20.2 per cent; DOMA (3,4-dihydroxymandelic acid) and VMA (vanillyl mandelic acid) constituted approximately 17 per cent each and NMN (normetanephrine) only 4 per cent. Thus, deaminated compounds (DOPEG, DOMA) formed approximately 51 per cent of the basal efflux, the o-methylated deaminated compounds (MOPEG, VMA) approximately 38 per cent, and o-methylated compounds (NMN) approximately 4 per cent. When TNS was applied at 2 Hz a sustained contraction resulted. This was accompanied by an increase in total radioactivity, ^{3}H-noradrenaline and the metabolites with the total fractional release amounting to 4.97 (mean ± 0.82, SEM). The (unmetabolised) ^{3}H-noradrenaline constituted 26.2 per cent of the total radioactivity (fractional release, mean 1.3 ± 0.12 SEM) with MOPEG, VMA, NMN and DOMA constituting approximately 23.3, 7.7, 18.3 and 10.3 per cent respectively. Fig. 36 shows a representative example from Protocol 2.1 and the summary data for total radioactivity and the different metabolite fractions during the experiments (n=5). After 14 minutes of TNS, exogenous noradrenaline was introduced into the superfusing fluid in a concentration of 10^{-6} mol/l while maintaining the TNS at 2 Hz for 14 minutes. This resulted in a further increase of tension accompanied by a decrease in the efflux of total radioactivity as well as ^{3}H-noradrenaline. The fractional release of total radioactivity was 2.60 (mean ± 0.33 SEM) while that of ^{3}H-noradrenaline was 0.36 (mean ± 0.06 SEM) during this period. At the end of the 14 minutes the exogenous noradrenaline was removed from the superfusing fluid while maintaining the TNS. This resulted in an increase in the fractional release of total radioactivity (mean 4.75 ± 0.84 SEM) and ^{3}H-noradrenaline (mean 1.11 ± 0.11 SEM) to levels close to that observed prior to the introduction of exogenous noradrenaline. After another 14 minutes the TNS was terminated with a resultant decrease in the efflux of tritiated compounds to basal levels. At the end of this period the fractional release of total radioactivity was 1.48 (mean ± 0.19, SEM) and that of ^{3}H-noradrenaline was 0.06 (mean ± 0.01 SEM) which were comparable to the basal efflux at the commencement of the experiment.

Thus, the introduction of exogenous noradrenaline into the superfusing fluid during TNS, resulted in a decrease in the efflux of total radioactivity as well as ^{3}H-noradrenaline induced by the TNS. The efflux produced by TNS in the presence of exogenous noradrenaline was compared with the mean efflux during the two periods of TNS before and

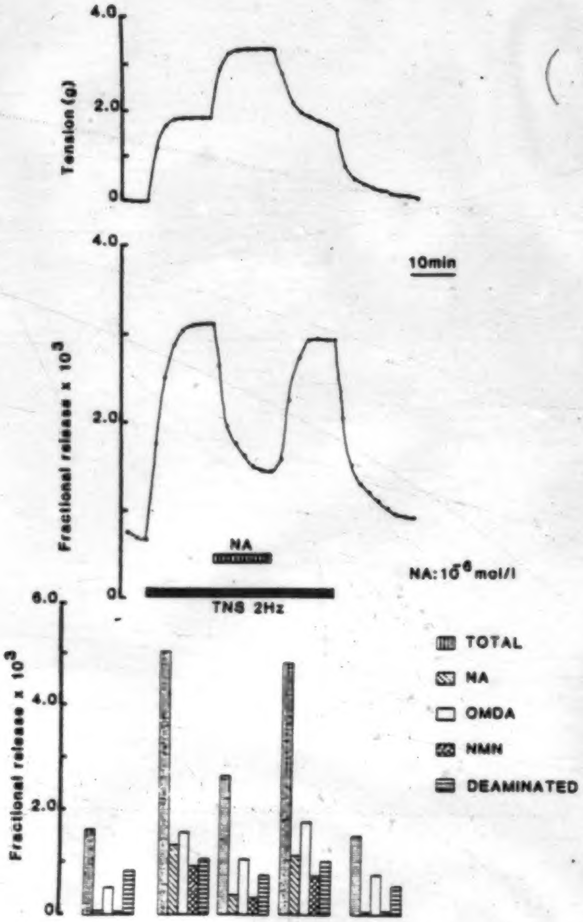


Figure 36. Summary of results from Protocol 2.1 The effect of exogenous noradrenaline added against a background of transmural nerve stimulation (TNS), on the total radioactivity of superfusate and efflux of ^{3}H-noradrenaline and metabolites produced by TNS (bottom) (refer Table 5 for standard errors of the mean). An example from Protocol 2.1 is shown also. Changes in tension (top) and total radioactivity in the superfusate (middle) during the experiment are shown. All radioactivity measurements are expressed as fractional release x 10^3.
NA:unmetabolised noradrenaline, Deaminated:deaminated metabolites (DOMA and DOPEG), OMDA:o-methylated deaminated metabolites (MOPEG and VMA), NMN:normetanephrine, TOTAL:Total radioactivity in the superfusate.

after the introduction of exogenous noradrenaline (NA) using a paired t-test. The results are summarised below as fractional release (mean ± SEM, n=5).

Total radioactivity

TNS only	4.85 ± 0.84	$p<0.001$
TNS + NA	2.60 ± 0.33,	
Ratio	0.558	

^{3}H-noradrenaline

TNS only	1.20 ± 0.11	$p<0.01$
TNS + NA	0.36 ± 0.06	
Ratio	0.300	

Evoked release was also calculated for each period of stimulation during the protocol (evoked release is the efflux during a period of intervention minus the basal efflux, i.e., the increase in efflux produced by the intervention itself). Comparisons made using evoked release values (instead of the total release as given above) too demonstrated the inhibition of the efflux of total radioactivity and ^{3}H-noradrenaline by exogenous noradrenaline.

The results of this protocol indicated that exogenous noradrenaline significantly inhibited the efflux of total radioactivity as well as ^{3}H-noradrenaline produced by TNS. All the data from the column chromatographic analysis during the present protocol are summarised in Table 3.

EXPERIMENTAL PERIOD	TOTAL RADIOACTIVITY	NA	MOPEG	VMA	NMN	DOMA	DOPEG
I CONTROL	1.58±0.10*	0.07±0.01	0.32±0.02	0.28±0.03	0.06±0.01	0.26±0.01	0.54±0.02
		(4.3)	(20.2)	(18.0)	(4.0)	(16.7)	(34.3)
II TNS	4.97±0.82	1.30±0.12	1.16±0.05	0.38±0.02	0.91±0.11	0.51±0.03	0.56±0.10
		(26.2)	(23.3)	(7.7)	(18.3)	(10.3)	(11.4)
III TNS	2.60±0.33	0.36±0.06	0.66±0.01	0.38±0.03	0.32±0.03	0.28±0.07	0.48±0.06
+ NA		(14.0)	(25.5)	(14.7)	(12.1)	(10.8)	(18.3)
IV TNS	4.75±0.84	1.11±0.11	1.20±0.05	0.54±0.05	0.73±0.09	0.43±0.03	0.56±0.11
		(23.4)	(25.3)	(11.4)	(15.4)	(9.1)	(11.9)
V CONTROL	1.48±0.19	0.06±0.01	0.34±0.01	0.41±0.05	0.07±0.07	0.18±0.01	0.37±0.04
		(4.0)	(23.0)	(27.5)	(4.6)	(11.8)	(25.0)

Table 3: Summary of results of column chromatographic analysis of samples from Protocol 2.1

TNS: Transmural nerve stimulation, 2 Hz; NA: Exogenous noradrenaline, 10^{-6} mol/1; *Mean fractional release ± SEM; ** per cent of total radioactivity; ***Recovery of radioactivity for all samples during the analysis = 96.5 per cent (mean ± 0.64% SEM).

Protocol 2.2

Step C, Step D and Step E of Protocol 1.2 were carried out here as a superfusion experiment. The protocol proper was begun two hours after the commencement of the superfusion when the basal efflux of the tritiated material had come down to a steady, low level. The fractional release of total radioactivity at this time was 2.06 (mean ± 0.20 SEM). The percentages of the different metabolite fractions contained in the total radioactivity were similar to the values obtained (at the commencement of the experiment proper) in Protocol 2.1; NA: 4.2 per cent (fractional release: mean 0.09 ± 0.01 SEM) MOPEG: 18.1 per cent VMA: 16.3 per cent, NMN: 3.5 per cent, DOMA: 18.2 per cent, DOPEG: 35.1 per cent. A representative example from the present protocol is shown in Fig. 37 together with the summary data for total radioactivity and the metabolite fractions during the experiments (n=5). After the control period, TNS was applied at 3 Hz. This produced an increase in the fractional release of total radioactivity (mean 5.49 ± 0.80 SEM) as well as ^{3}H-noradrenaline (mean 1.44 ± 0.08, SEM). The latter constituted approximately 26.3 per cent of the total radioactivity during this period. TNS was terminated after 14 minutes and a control period followed, during which the efflux of tritiated compounds returned to basal levels (total radioactivity: mean 1.65 ± 0.08 SEM, ^{3}H-noradrenaline 4.1 per cent of total). Exogenous noradrenaline in a concentration of 3.3 x 10^{-7} mol/l was introduced into the superfusing solution after this control period, for 14 minutes. This resulted in an increase in tension but it did not change the total efflux or the relative proportions of the metabolite fractions to any appreciable extent. The total fractional release during this period was 1.45 (mean

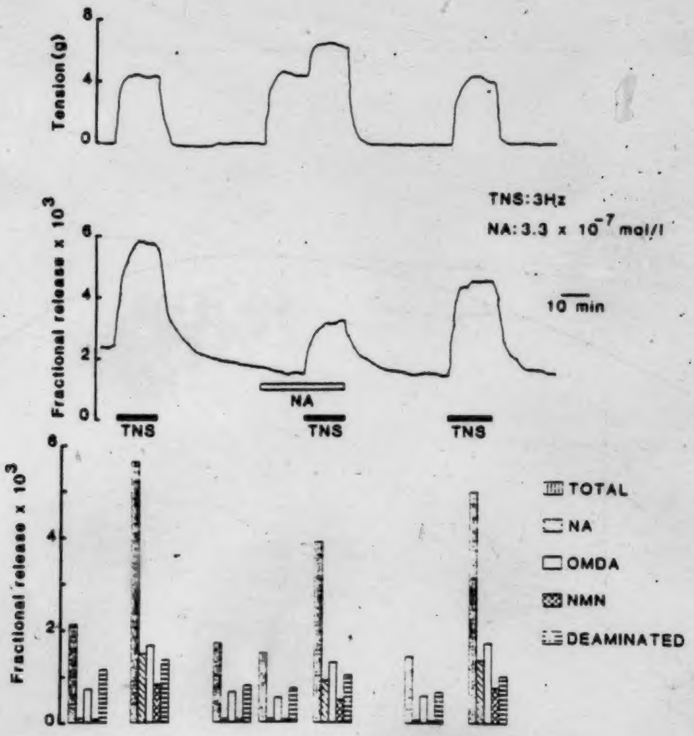


Figure 37. Summary of results from Protocol 2.2. The effect of background exogenous noradrenaline on the total radioactivity of the superfusate and efflux of ^{3}H-noradrenaline and metabolites (bottom) (refer Table 6 for standard errors of the mean). An example from Protocol 2.2 is shown also. Changes in tension (top) and total radioactivity in the superfusate (middle) during the experiment are shown. All radioactivity measurements are expressed as fractional release x 10^3. NA:unmetabolised noradrenaline, Deaminated:deaminated metabolites (DOMA and DOPEG), OMDA:o-methylated deaminated metabolites (MOPEG and VMA), TNS:transmural nerve stimulation, NMN:normetanephrine, TOTAL:total radioactivity in the superfusate.

± 0.07 SEM) and the ^{3}H-noradrenaline release was 0.07 (mean ± 0.01 SEM). At the end of 14 minutes TNS was applied (at the same frequency as used before) while continuing the superfusion with fluid containing exogenous noradrenaline. This produced a further increase in tension accompanied by an increase in the efflux of tritiated compounds. The total radioactivity present in the superfusate during this period was 3.81 (mean ± 0.89, SEM) and the ^{3}H-noradrenaline content 0.89 (mean ± 0.10 SEM: 23.4 per cent of total). The relative proportions of the different metabolites were similar to that observed during application of TNS in the absence of a background contraction produced by exogenous noradrenaline. At the end of this period (14 minutes) TNS was terminated and the superfusing solution changed back to one without exogenous noradrenaline. The efflux of tritiated material fell to basal levels during this period: total radioactivity:mean 1.39 ± 0.08 SEM and ^{3}H-noradrenaline:mean 0.06 ± 0.01 SEM. TNS applied after this control period produced an increase in tension accompanied by an enhanced efflux of tritiated material. The total efflux of radioactivity during this period was 4.86 (mean ± 0.77 SEM) and the ^{3}H-noradrenaline efflux 1.31 (mean ± 0.05 SEM, 26.9 per cent of total). TNS was terminated at the end of 14 minutes resulting in a fall of the efflux of tritiated material to basal levels.

During this protocol, both the total efflux and the ^{3}H-noradrenaline efflux produced by TNS in the presence of exogenous noradrenaline was less than that produced in its absence. The means of the efflux values during the two periods of TNS (in the absence of exogenous noradrenaline) were compared with the efflux produced by TNS in the presence of exogenous noradrenaline by a paired t-test. The results are summarised below as fractional release (mean ± SEM, n=5).

Total radioactivity

TNS only	5.07 ± 0.79	$p<0.05$
NA + TNS	3.81 ± 0.89	
Ratio	0.73	

^{3}H-noradrenaline

TNS only	1.35 ± 0.06	$p<0.05$
NA + TNS	0.89 ± 0.10	
Ratio	0.66	

Comparison of the same using evoked fractional release (i.e., efflux during an intervention minus the basal efflux) demonstrated similar results. All the data from the column chromatographic analysis are summarised in Table 4.

Therefore, in both Protocol 2.1 and Protocol 2.2 significant inhibition of the TNS induced efflux of tritiated compounds including unmetabolised ^{3}H-noradrenaline occurred in the presence of exogenous noradrenaline in the superfusing solution. Evoked release of tritiated compounds during TNS in the presence of exogenous noradrenaline was expressed as a percentage of that in the absence of the exogenous drug (evoked fractional release was utilised as it reflects the efflux induced by TNS more accurately). This was done for each individual experiment in both protocols. These percentages (which reflect inversely the degree of inhibition produced by exogenous noradrenaline) from the two protocols were compared by a students t-test for unpaired data. The mean percentage for Protocol 2.1 was 32.9 per cent (SEM 2.7%) and for Protocol 2.2 was 57.6 per cent (SEM 9.9%). These two values were significantly different from each other ($p<0.05$).

EXPERIMENTAL PERIOD	TOTAL RADIOACTIVITY	NA	MOPEG	VMA	NMN	DOMA	DOPEG
I CONTROL	2.06±0.20*	0.09±0.01 (4.2)	0.37±0.03 (18.1)	0.33±0.06 (16.2)	0.07±0.01 (3.5)	0.38±0.02 (18.2)	0.72±0.06 (35.1)
II TNS	5.49±0.80	1.44±0.08 (26.3)	1.19±0.07 (21.6)	0.42±0.05 (7.6)	0.80±0.04 (14.5)	0.62±0.04 (11.3)	0.69±0.12 (12.5)
III CONTROL	1.65±0.08	0.07±0.02 (4.1)	0.29±0.02 (17.4)	0.35±0.06 (21.4)	0.08±0.02 (4.9)	0.29±0.02 (17.3)	0.49±0.04 (29.7)
IV NA	1.45±0.07	0.07±0.01 (4.61)	0.25±0.01 (16.9)	0.27±0.06 (18.8)	0.06±0.01 (4.2)	0.24±0.02 (16.5)	0.51±0.04 (35.3)
V NA + TNS	3.81±0.89	0.89±0.10 (23.4)	0.92±0.05 (24.2)	0.35±0.05 (9.2)	0.49±0.04 (12.9)	0.45±0.05 (11.8)	0.59±0.10 (15.4)
VI CONTROL	1.39±0.08	0.06±0.01 (4.1)	0.27±0.02 (19.2)	0.30±0.06 (21.3)	0.05±0.01 (3.4)	0.21±0.02 (14.9)	0.45±0.05 (32.1)
VII TNS	4.86±0.77	1.31±0.05 (26.9)	1.32±0.16 (27.1)	0.35±0.03 (7.3)	0.74±0.04 (15.3)	0.50±0.04 ~~(10.3)~~	0.50±0.07 (10.2)

Table 4 : Summary of results of column chromatographic analysis of samples from Protocol 2.2

TNS: Transmural nerve stimulation, 3 Hz; Na:Exogenous noradrenaline $3.3\text{x}10^{-7}$ mol/1; *Mean fractional release ± SEM; ** per cent of total radioactivity; ***Recovery of radioactivity for all samples during the analysis = 95.7 per cent (mean ± 0.68% SEM)

Protocol Three

A possible explanation for the inhibition of the exogenous noradrenaline contraction by TNS in Protocol 1.1 is a concurrent relaxatory response by TNS. The present protocol was an attempt to demonstrate the existence of such a relaxatory response in the canine saphenous veins, following blockade of the contractile response to TNS.

Saphenous vein rings responded to transmural nerve stimulation with frequency dependent contractions which were maximum at a frequency of 16-32 Hz. When the stimulus-response curves were repeated (in preliminary experiments) following incubation of the vein rings with tetrodotoxin (10^{-6} mol/l) for 30 minutes, the contractile response to TNS was almost completely abolished (Fig. 38). The maximum contraction at 32 Hz in the presence of tetrodotoxin was 3.4 per cent (mean ± 1.7 per cent SEM, n=5) of the control value. This confirmed that the contractile response to the TNS (applied as square-wave pulses 1.0 ms duration, 10 V strength) was due to the activation of intramural nerves. In the experiment proper, blockade of this contractile response to TNS was achieved by a combination of guanethidine and phenoxybenzamine. Following the addition of guanethidine into the tissue bath an increase in tension was observed in the vein rings. The magnitude of this increase varied considerably from preparation to preparation. Some preparations demonstrated intermittent contractions superimposed on the increased tone. At the end of 90 minutes when phenoxybenzamine (2×10^{-5} mol/l) was added, the tension returned to the basal value and remained relatively stable thereafter. After another 30 minutes TNS was applied at a frequency of 32 Hz to ensure adequate sympathetic blockade. At this time the response to TNS was almost completely abolished in most vein rings.

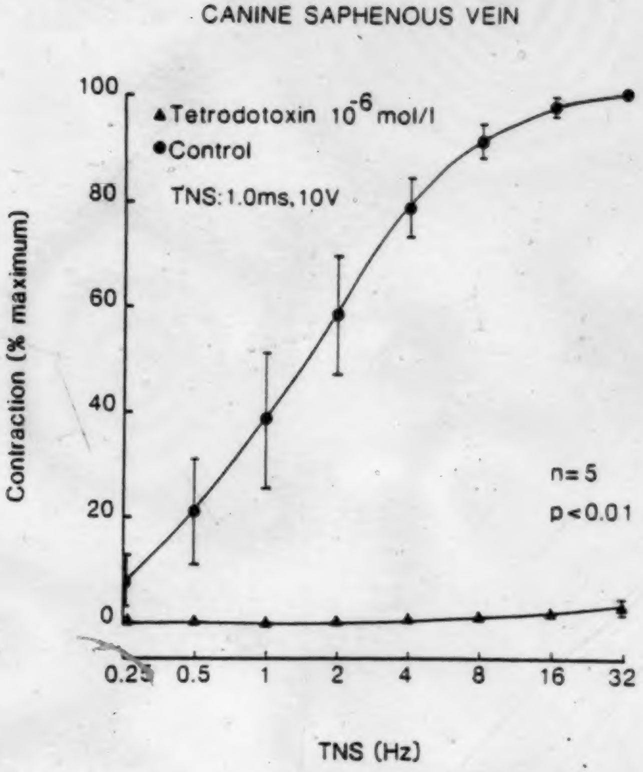


Figure 38: The effect of tetrodotoxin (10^{-6} mol/l) on the contraction to transmural nerve stimulation (TNS) in the isolated canine saphenous vein. TNS: square wave pulses: duration 1.0 ms, strength 10V, frequency 0.25 to 32 HZ.

Protocol 3.1

The basal tension in the vein rings was 2.95 g (mean ± 0.19 g SEM) at the commencement of the experiment proper (n=17). Following the blockade of the contractile response to TNS, the rings were made to contract by the addition of prostaglandin $F_{2\alpha}$ (10^{-5} mol/l). The contraction reached a plateau after 10-15 mintues. The tension at this time was 17.53 g (mean ± 1.27 g SEM, n=17). TNS applied at this stage as intermittent trains of stimuli (30 s duration) at each frequency (1-32 Hz), elicited a frequency dependent relaxation in the rings (Fig. 39A). Relaxation commenced within a few seconds of the commencement of TNS. Complete recovery from the relaxation (following cessation of TNS) occurred within 5-10 mintues, although in some instances, especially at higher frequencies, it took considerably longer (up to 30 minutes). Further, at the higher frequencies a second, delayed, relaxation was sometimes observed when the TNS was terminated at the end of 30 seconds. Thus, the response consisted of two phases: an initial transient relaxation and a second, delayed, relaxation with slow recovery (Fig. 39B). The second phase (when observed) was sometimes of greater magnitude than the initial phase. The magnitude of the relaxation, in general, varied considerably from tissue to tissue. The relaxation was observed to be small in preparations in which the contractile response to TNS was not completely abolished by the guanethidine/phenoxybenzamine pre-treatment. All the data from the frequency response curves are summarised in Fig. 40. The maximum relaxation observed was 3.28 g (mean ± 0.18 g SEM, n=17).

In order to determine whether the relaxation observed was mediated through the endothelium the experiment was carried out in rings

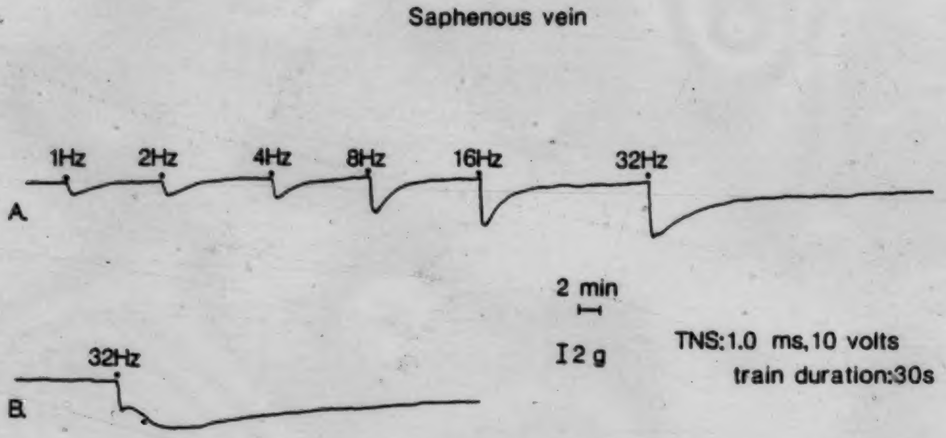


Figure 39. Response of canine saphenous vein rings, pre-contracted with prostaglandin $F_{2\alpha}$ (10^{-5} mol/l), to transmural nerve stimulation (TNS) following sympathetic and muscarinic blockade using guanethidine (10^{-4} mol/l), phenoxybenzamine (2×10^{-5} mol/l), propranolol (2×10^{-6} mol/l) and atropine (5×10^{-6} mol/l). TNS applied as intermittent 30 second trains of stimuli at each frequency. A. demonstrating a frequency dependent relaxation to intermittent trains of stimuli. B: example of a biphasic response to TNS at 32 Hz demonstrating an initial transient relaxation and a second delayed after-relaxation following cessation of TNS. Note: slow recovery from the relaxation.

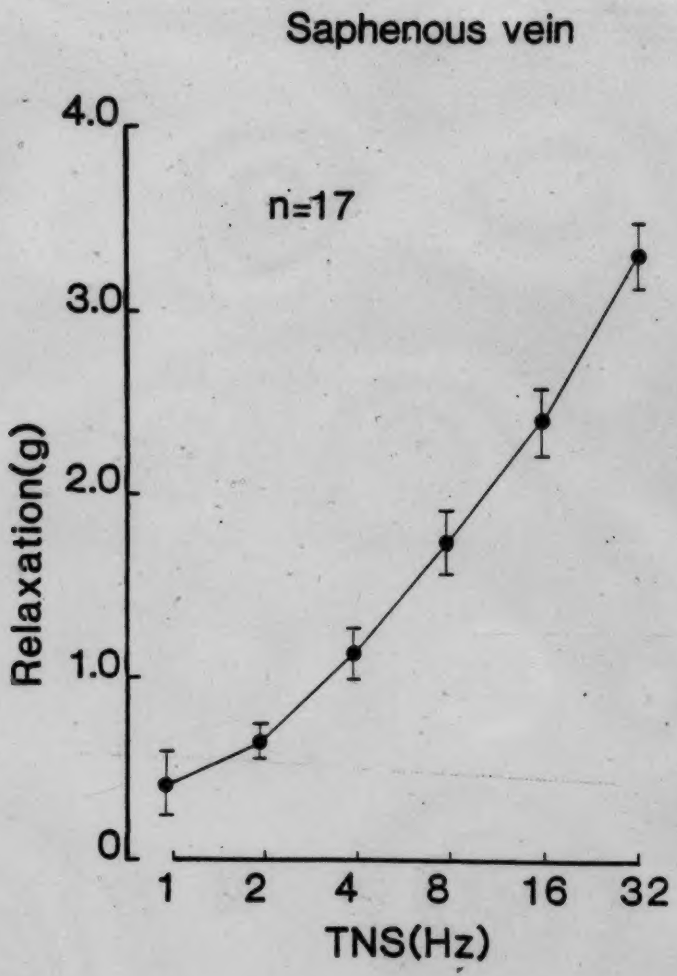


Figure 40. The relationship of the magnitude of the relaxation to transmural nerve stimulation (TNS) to frequency of TNS, in canine saphenous vein rings pre-contracted with prostaglandin $F_{2\alpha}$ (10^{-5} mol/l) following sympathetic and muscarinic blockade. TNS:trains of 30 second duration at each frequency, pulse duration: 1.0 ms, strength:10 V.

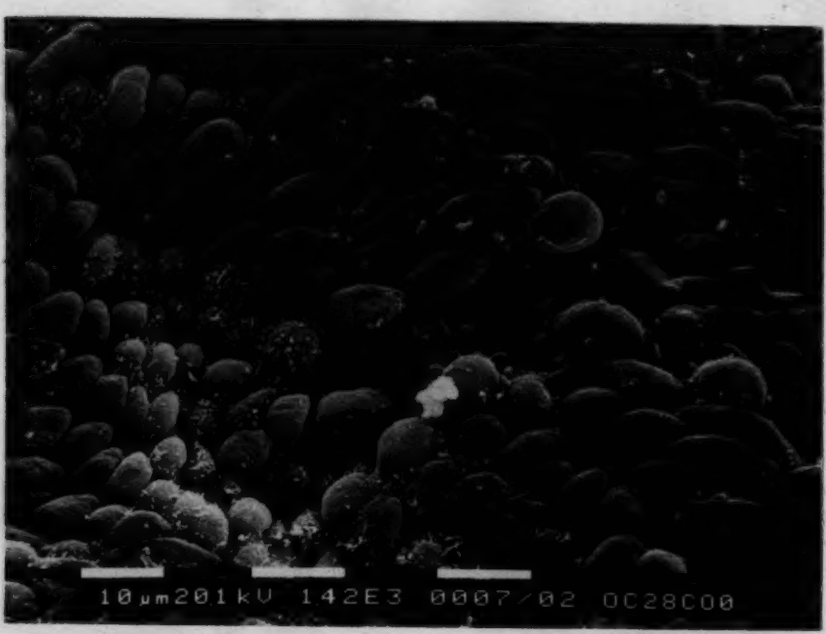
mechanically denuded of endothelium, (as described under Methods) with parallel controls. Confirmation of the preservation of the endothelium in the control rings and the removal of the endothelium by the mechanical denudation was obtained by the use of scanning electron microscopy. The rings were removed at the end of the experiments and carefully cut open to be fixed and processed for scanning electron microscopy of their luminal surfaces. Two electron-micrographs from control and de-endothelialised rings are shown in Fig. 41. As can be seen, the ring mechanically denuded of the endothelium showed no evidence of intact endothelium. On the other hand, the endothelium was preserved in 80-90 per cent of the luminal surface in control rings.

The optimum basal tension for the rings mechanically denuded of endothelium (mean; 2.60g ± 0.16 g SEM, n=12) was not significantly different from the controls (mean; 2.75g ± 0.18g SEM, n=12) ($p>0.05$). Further, the response of these rings to prostaglandin $F_{2\alpha}$ (maximum tension: mean; 17.90g ± 1.33g SEM) was also not significantly different from the controls (maximum tension: mean 18.77 g ± 1.45g SEM) ($p>0.05$, n=12). TNS applied to these rings elicited a frequency dependent relaxation. The data are summarised in Fig. 42. The maximum relaxation observed was 3.50 g (mean ± 0.42g SEM, n=12) in the de-endothelialised rings and 3.40g (mean ± 0.21g SEM, n=12) in the control rings. The stimulus-response curves compared by regression analysis with analysis of covariance (See under statistical Methods) did not show a significant difference ($p>0.05$).

When TNS was applied continuously, in a "cumulative" fashion at frequencies of 1 through 32 Hz (instead of intermittent trains of stimuli for 30 seconds at each frequency) the pattern of relaxation was

Figure 41: A. Scanning electron micrograph of the intimal surface of a saphenous vein ring fixed and processed at the end of an experiment. Approximately 80 per cent of the intimal surface has endothelial cells. White bars at the bottom = 10 μm. Magnification:1.42 x 10^3. B. Scanning electron micrograph of the intimal surface of a vein ring mechanically denuded of the endothelium. White bars = 100 μm Magnification:1.43 x 10^2. Note: absence of endothelial cells.

A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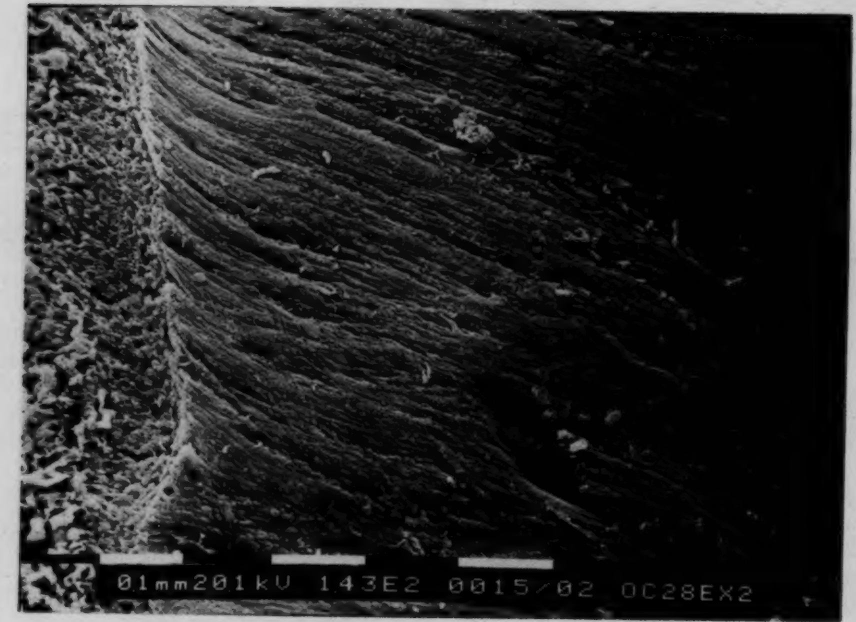
B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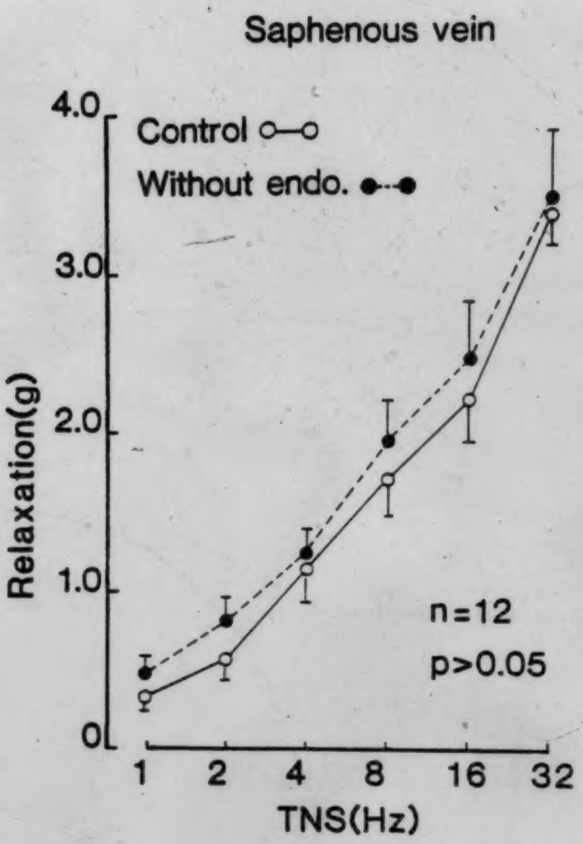

Figure 42. The effect of mechanical de-endothelialisation on the relaxatory response to transmural nerve stimulation in canine saphenous vein rings pre-contracted with prostaglandin $F_{2\alpha}$ (10^{-5} mol/l) following sympathetic and muscarinic blockade. TNS:trains of 30 second duration at each frequency.

inconsistent, with a definite frequency dependent relaxation being observed only occasionally (Fig. 43). All rings showed a relaxatory response at 1 Hz. When the frequency of stimulation was increased, there was no further relaxation from 2-8 Hz in most rings. In some preparations the tension returned towards the baseline during these intermediate frequencies. At higher frequencies of stimulation (16-32 Hz) most preparations responded with further relaxations. Nevertheless, the maximum relaxation observed at 32 Hz with this mode of stimulation appeared to be less than that observed during a 30 second train of stimuli at 32 Hz. Following continuous stimulation, some preparations showed a delayed relaxation, the recovery from which was prolonged, occasionally taking up to 30 minutes. It was also incomplete at times. Since the responses elicited by intermittent trains of stimuli (30 second) were more reproducible than those elicited by continuous stimulation, all drug effects were tested against intermittent trains of stimuli.

Protocol 3.2

The effect of different drugs on the relaxatory response to TNS was investigated here. A stimulus-response curve to TNS (applied as intermittent 30 trains) was carried out first and then repeated in the presence of the drug.

TNS applied in the presence of the fast Na^+ channel inhibitor tetrodotoxin (10^{-6} mol/l) elicited a frequency dependent relaxation. The maximum relaxation in the presence of tetrodotoxin was 2.62 g (mean ± 0.20 g SEM, n=6) and in its absence was 2.77 g (mean ± 0.25 g SEM, n=6). The results are summarised in Fig. 44. The stimulus-response curves with and without tetrodotoxin were not significantly different

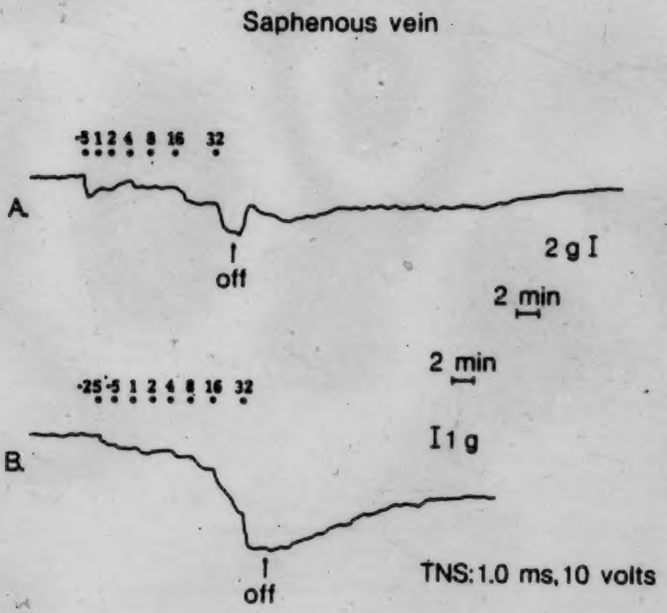

Figure 43. Response of canine saphenous vein rings pre-contracted with prostaglandin $F_{2\alpha}$ (10^{-5} mol/l), to transmural nerve stimulation (TNS) following sympathetic and muscarinic blockade. Stimulus-response curves to TNS done "cumulatively" using continuous stimulation (in contrast to the protocol shown in Fig. 39). A. TNS at 0.5 Hz produced a relaxation which was reversed partially with an increase in the frequency of stimulation (i.e. 1 to 8 Hz). However at the higher frequencies (16 to 32 Hz) the relaxation was evident again. B. Example of a more definite frequency dependent relaxation with continuous TNS:such a response was seldom observed with this mode of stimulation.

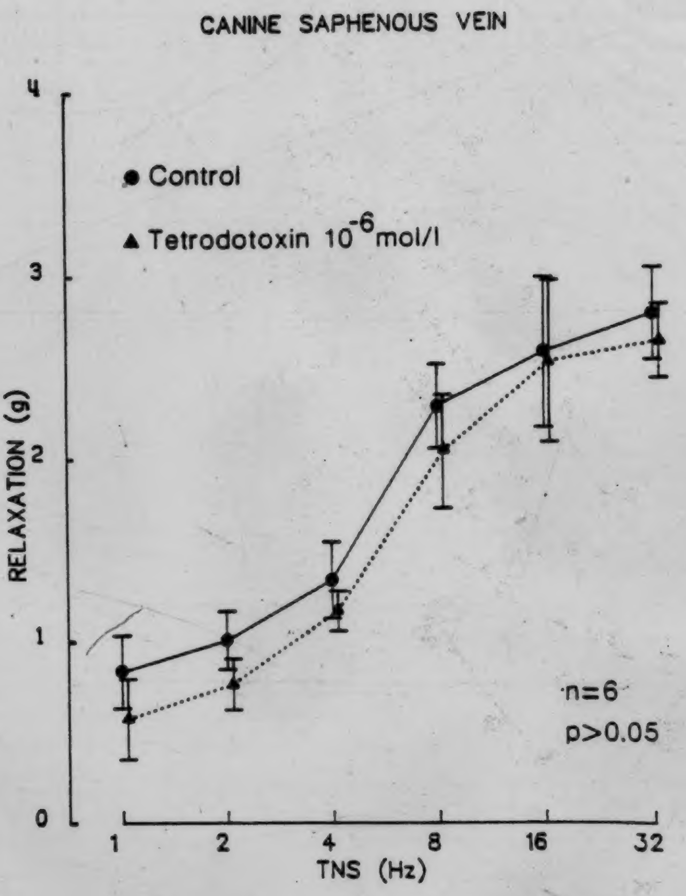

Figure 44. Effect of tetrodotoxin on the relaxatory response to transmural nerve stimulation (TNS) in canine saphenous vein rings precontracted with prostaglandin $F_{2\alpha}$ following sympathetic and muscarinic blockade. TNS:trains of 30 second duration at each frequency.

from each other ($p>0.05$). As mentioned before, this concentration of tetrodotoxin almost completely abolished the contractile response to TNS (prior to application of guanethidine and phenoxybenzamine) in the saphenous vein.

Incubation with the H_2-receptor antagonist cimetidine in a concentration of 10^{-4} mol/l did not antagonise the relaxatory response to TNS. The maximum relaxation in the presence of cimetidine was 2.88 g (mean ± 0.53 g SEM, n=5) and the maximum relaxation in the control preparations was 2.92 g (mean ± 0.47 g SEM, n=5). The pooled data from the stimulus-response curves are shown in Fig. 45. There was no significant difference between the two curves ($p>0.05$, n=5).

Indomethacin in a concentration of 10^{-5} mol/l failed to modify the relaxatory response to TNS. The maximum relaxation with and without indomethacin were 2.82 g (mean ± 0.40 g SEM) and 3.23 g (mean ± 0.38 g SEM) respectively (n=6). The pooled data shown in Fig. 45 demonstrated no significant difference between the two stimulus-response curves ($p>0.05$, n=6).

In the experiments with the P_2-receptor antagonist aminophylline the drug was used in a concentration of 10^{-5} mol/l. At this concentration it had no effect on the contraction produced by the prostaglandin $F_{2\alpha}$. The maximum relaxation to TNS in the presence of aminophylline was 2.82 g (mean ± 0.49 g SEM, n=6) and that in its absence was 3.08 g (mean ± 0.39 g SEM, n=6). The pooled data are shown in Fig. 46. There was no significant difference between the stimulus-response curves with and without aminophylline ($p>0.05$, n=6).

The introduction of ouabain into the tissue-bath fluid in a concentration of 2×10^{-4} mol/l resulted in a gradual increase of the

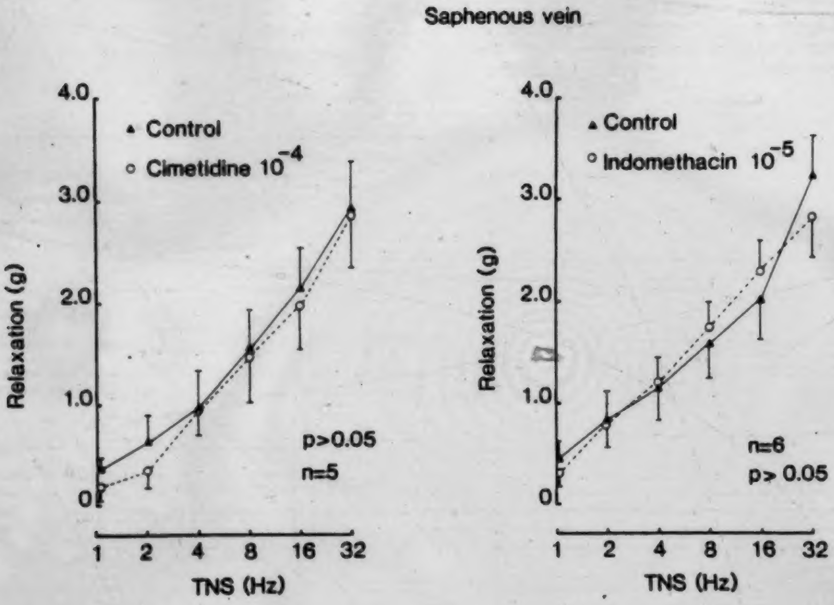

Figure 45. Effect of cimetidine (left) and indomethacin (right) on the relaxatory response to transmural nerve stimulation (TNS) in canine saphenous vein rings pre-contracted with prostaglandin $F_{2\alpha}$ (10^{-5} mol/l) following sympathetic and muscarinic blockade. TNS:trains of 30 second duration at each frequency. The drug concentrations are given in mol/l.

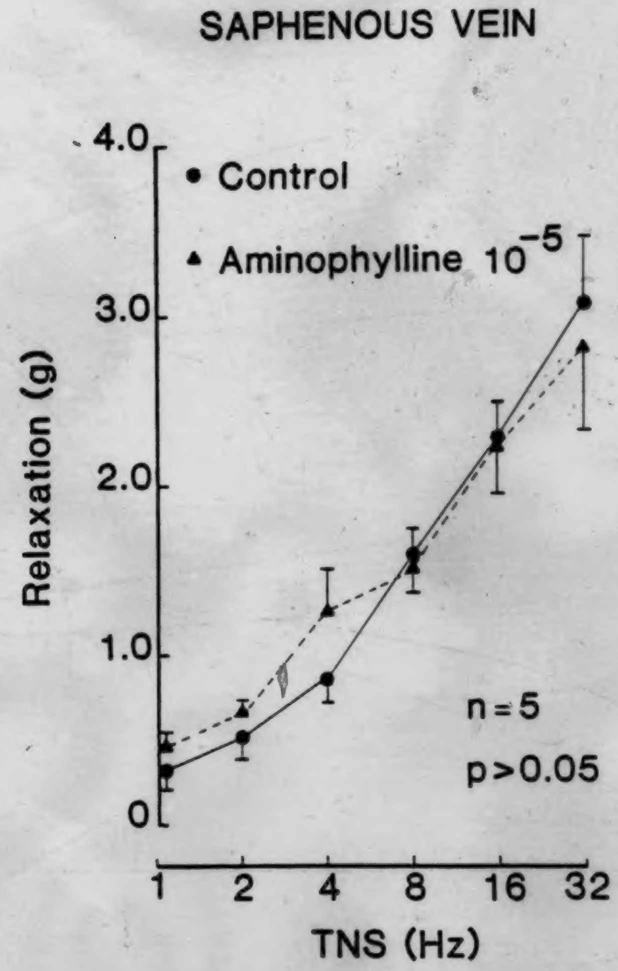

Figure 46. Effect of aminophylline (10^{-5} mol/l) on the relaxatory response to transmural nerve stimulation (TNS) in canine saphenous vein rings pre-contracted with prostaglandin $F_{2\alpha}$ (10^{-5} mol/l) following sympathetic and muscarinic blockade. TNS:trains of 30 second duration at each frequency.

active tension produced by the prostaglandin $F_{2\alpha}$ in some rings. This increase varied from 0-15 per cent of the active tension prior to the introduction of the drug. The relaxatory response to TNS was almost abolished in the presence of ouabain (the effect appeared to be similar regardless of the presence or absence of increased active tension induced by ouabain). The maximum relaxation in the presence of the drug was 0.55 g (mean ± 0.09 g SEM, n=6) and that in its absence 2.73 g (mean ± 0.24 g SEM, n=6). The decrease in the response was much more evident at the lower frequencies with practically no relaxation being observed up to (and including) a frequency of 8 Hz (Fig. 47). The two stimulus-response curves were significantly different from each other ($p<0.001$, n=6).

In the experiments using "zero-K^+ Krebs buffer" solution a marked decrease in active tension was observed when the normal Krebs buffer solution in the tissue bath was replaced by the above solution, in spite of the continued presence of of prostaglandin $F_{2\alpha}$ in the bath. The active tension fell to 15-35 per cent of the control value (mean; 23.75 per cent) after 30 minutes of incubation in the zero-K^+ Krebs buffer. TNS applied at the end of this period failed to produce any significant relaxation. The maximum relaxation observed in zero-K^+-Krebs buffer was 0.26g (mean ± 0.13g SEM, n=5) while that in the controls was 2.08g (mean ± 0.3g SEM, n=5). The pooled stimulus-response curves was significantly different from each other (Fig. 47, $P<0.01$, n=5).

A possible role for free radicals in the observed relaxatory response to TNS was investigated by using ascorbic acid (10^{-4} mol/l) and the enzyme catalase (50 μg/ml). Ascorbic acid is a non-specific scavenger of free radicals while the latter specifically scavenges

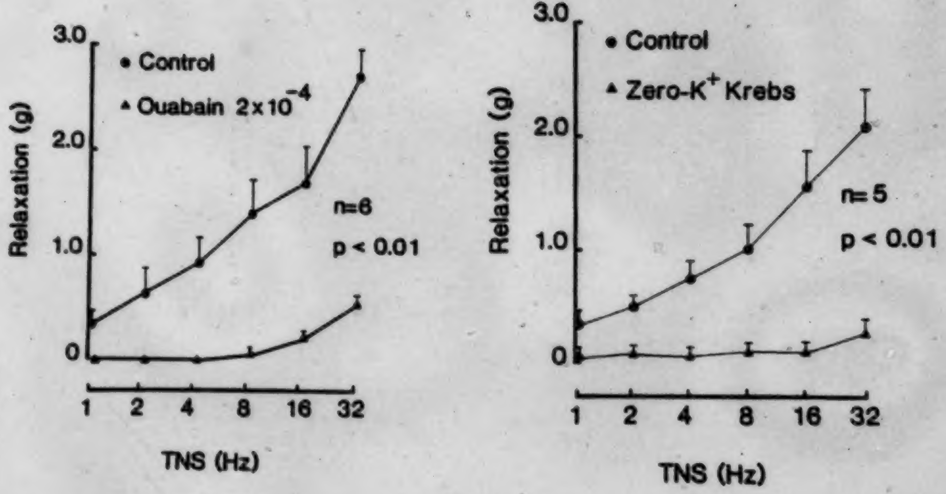

Figure 47. Effect of ouabain ($2x10^{-4}$ mol/l) and zero-K^+ "Krebs" buffer solution on the relaxatory response to transmural nerve stimulation (TNS) in canine saphenous vein rings pre-contracted with prostaglandin $F_{2\alpha}$ (10^{-5} mol/l) following sympathetic and muscarinic blockade. TNS: trains of 30 second duration at each frequency.

peroxide radicals. The two drugs by themselves did not alter the contractile responses to prostaglandin $F_{2\alpha}$. TNS applied in the presence of these drugs produced a frequency dependent relaxation as before. The maximum relaxation observed in the presence of ascorbic acid was 2.53 g (mean ± 0.19 g SEM, n=7) while that in its absence was 2.31 g (mean ± 0.25 g SEM, n=7). The stimulus-response curves constructed from the pooled data were not significantly different from each other (Fig. 48, $p>0.05$, n=7). The maximum relaxation to TNS in the presence of catalase and in the controls were 2.98 g (mean ± 0.43 g SEM) and 3.00 g (mean ± 0.52 g SEM) respectively (n=6). The pooled data did not show any significant difference between the stimulus-response curves with and without catalase (Fig. 48, $p>0.05$, n=6).

Protocol 3.3

The effect of storage of the isolated saphenous veins at 4°C for 9 days ("cold storage") in Krebs buffer solution, on the relaxatory response to TNS observed above was investigated in this protocol. The contractile response to the prostaglandin $F_{2\alpha}$ in these veins following cold storage was approximately 30-50 per cent of that observed in fresh rings taken from the same dogs. When isoprenaline (3×10^{-6} mol/l) was applied to these rings pre-contracted with prostaglandin $F_{2\alpha}$ (in the absence of the β-blocker, propranolol), the rings responded with relaxations; the tension produced by prostaglandin $F_{2\alpha}$ was 6.43 g (mean ± 0.67 g SEM) while the magnitude of the relaxation to isoprenaline was 6.37 g (mean ± 0.66 g SEM) (n=3). These results indicated that the rings of saphenous veins, even after cold storage for 9 days were capable of contraction and relaxation in response to exogenous agonists. However, the relaxatory response to TNS (applied to these

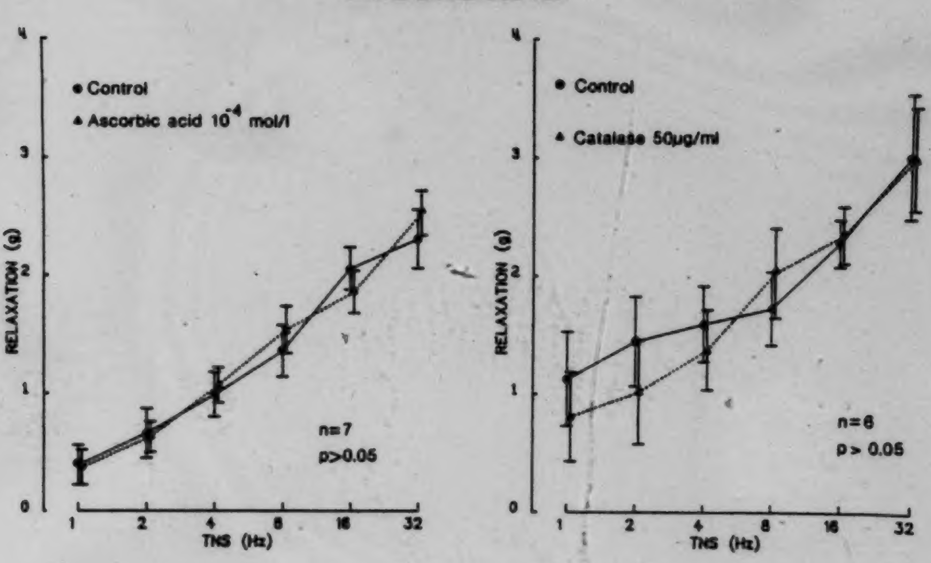

Figure 48. Effect of ascorbic acid (left) and catalase (right) on the relaxatory response to transmural nerve stimulation (TNS) in canine saphenous vein rings pre-contracted with prostaglandin $F_{2\alpha}$ (10^{-5} mol/l) following sympathetic and muscarinic blockade. TNS: trains of 30 second duration at each frequency.

rings following pre-contraction with prostaglandind $F_{2\alpha}$) was almost abolished in these veins. Experiments done from rings taken from the same veins on the day of excision (i.e., prior to cold storage) served as controls. The maximum relaxation observed in the venous rings following cold storage was 0.46 g (mean ± 0.23 g SEM) and that in the control rings was 3.12 g (mean ± 0.62 g SEM) (n=5). The pooled data are summarised in Fig. 49. The pooled stimulus-response curve in the venous rings following cold storage was significantly different from that in the controls ($p<0.01$, n=5). In some (three preparations) of these rings phenoxybenzamine and guanethidine too were applied to simulate the experimental conditions in the "fresh" rings. These rings too failed to show any relaxation to transmural nerve stimulation.

Protocol 3.4

The effect of acute chemical sympathetic denervation using 6-hydroxydopamine, on the relaxatory response to TNS was investigated in this protocol. There was an immediate increase in tension when the 6-hydroxydopamine was applied. This reached a peak in 4-5 minutes and gradually declined thereafter. With the second application of 6-hydroxydopamine after 10 minutes, a rise in tension was observed again. The tension returned to the basal level over the next 1-3 hours. The contractile response to TNS (at 32 Hz) was 10-20 per cent of the control value, 2 hours after the application of 6-hydroxydopamine, in most veins. At the end of 4 hours the contractile response was less than 5 per cent in almost all veins studied. Thus, the investigation of the TNS induced relaxation was carried out approximately 4 hours after the application of the 6-hydroxydopamine. Frequent rinsing of the tissue bath solution was carried out during this period. Rings (taken

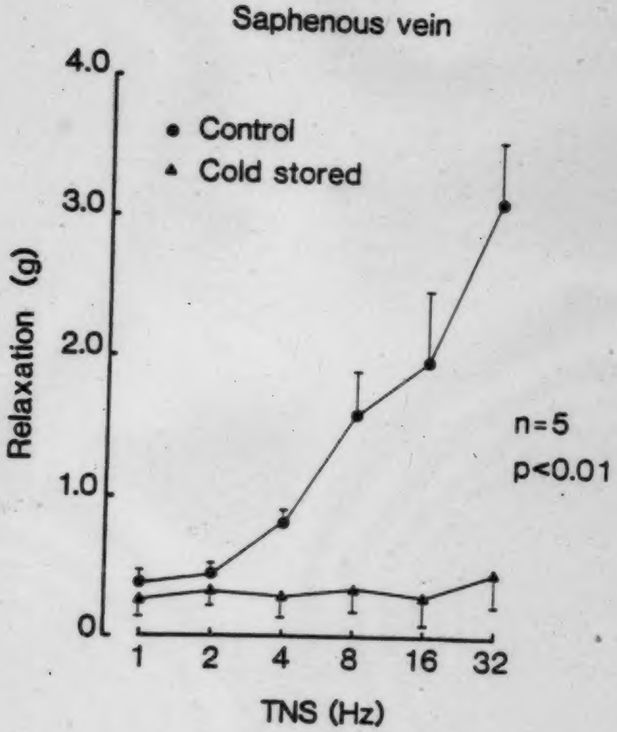

Figure 49. Effect of cold-storage of the isolated canine saphenous veins at 4°C for 9 days on the relaxatory response to transmural nerve stimulation (TNS) in the vein rings pre-contracted with prostaglandin $F_{2\alpha}$ (10^{-5} mol/l) following sympathetic and muscarinic blockade. TNS:trains of 30 second duration at each frequency.

from the same vein) without the application of 6-hydroxydopamine were used as controls. Guanethidine and phenoxybenzamine were used in these rings to block the contractile response to TNS. TNS applied following pre-contraction with prostaglandin $F_{2\alpha}$ elicited a frequency dependent relaxation in the 6-hydroxydopamine treated rings. The maximum relaxation observed was 3.2 g (mean ± 0.41 g SEM) in these rings. The maximum relaxation observed in the control rings was 2.88 g (mean ± 0.69g SEM, n=4). The pooled data are summarised in Fig. 50. The pooled stimulus-response curves with and without 6-hydroxydopamine treatment were not significantly different from each other ($p>0.05$, n=4).

Protocol Four

Five second trains of TNS applied at a frequency of 8 Hz (10 V, 0.3 ms) produced transient contractions with complete recovery in 60-120 seconds. With repeated trains of TNS (every 5 minutes) the response became stable after 5-10 applications. The response at this time had a mean of 2.61 g ± 0.40 g SEM (n=10). Low concentrations of noradrenaline added at this point produced a concentration dependent potentiation of the contractile response to the train of TNS (Fig. 51). This potentiation was evident within 30 seconds of the addition of the exogenous noradrenaline, and reached a maximum within 5-10 minutes. The potentiation of the response to TNS was still present even 30 minutes after the addition of exogenous noradrenaline. After removal of the noradrenaline from the tissue bath, the potentiation disappeared and the response to the train of TNS returned to the control value within 10-15 minutes. The highest concentration of exogenous noradrenaline utilised for this protocol was -7.2 $\log_{10}$ mol/l. At this concentration, noradrenaline produced a small contraction in some vein rings (mean

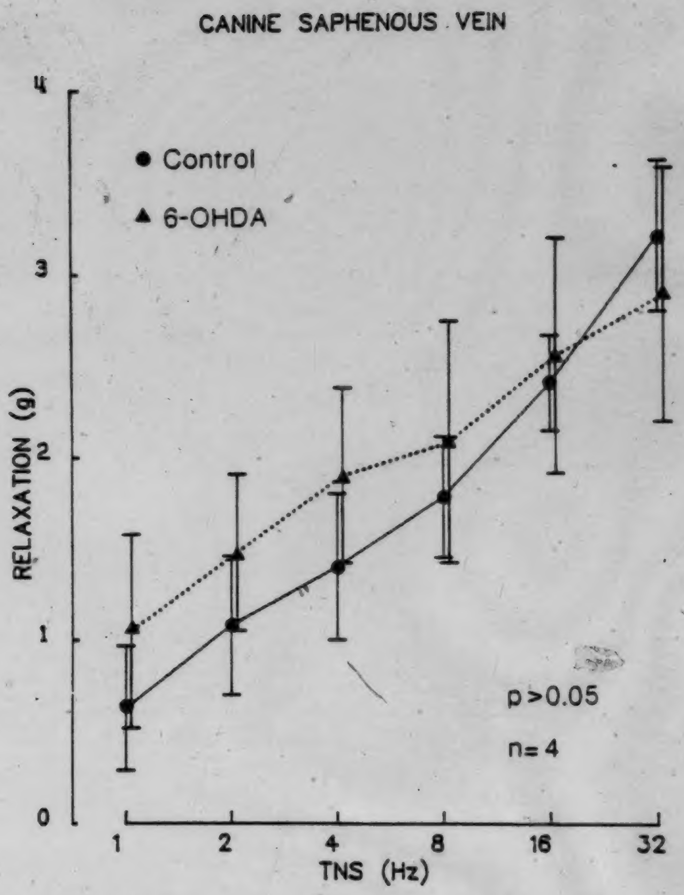

Figure 50. The effect of chemical sympathetic denervation with 6-hydroxydopamine (6-OHDA) on the relaxatory response to transmural nerve stimulation (TNS) in canine saphenous vein rings pre-contracted with prostaglandin $F_{2\alpha}$ (10^{-5} mol/l) following sympathetic and muscarinic blockade. TNS:trains of 30 second duration at each frequency.

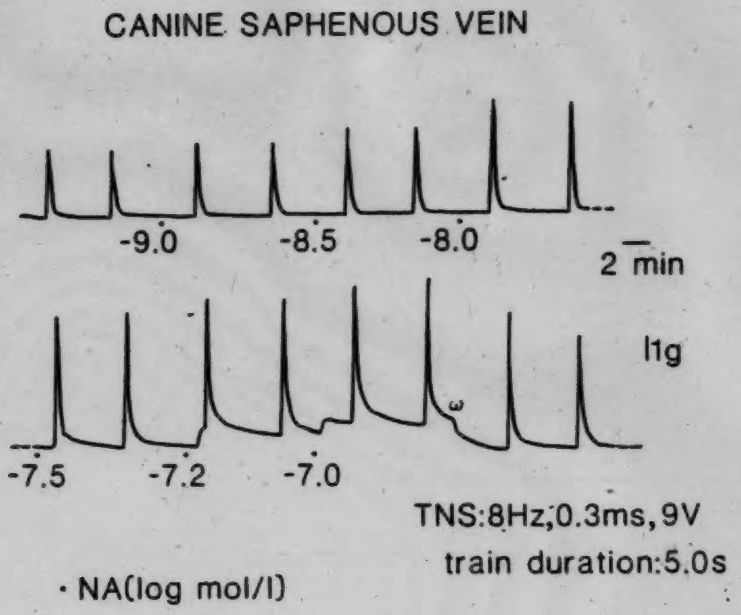

Figure 51. A representative example from Protocol Four. Trains of transmural nerve stimulation (TNS) were applied approximately every 5 minutes. Dots represent the points of addition of exogenous noradrenaline (NA) to the tissue bath cumulatively. A concentration dependent potentiation of the response to the train of TNS by exogenous noradrenaline NA is shown. W:rinsing of the tissue bath with fresh Krebs buffer solution.

0.34 g ± 0.15 g SEM, n=10). The response to the train of TNS at this concentration of noradrenaline was 246.2 per cent (mean ± 36.9 SEM, n=10) of the control. The responses to TNS at the different concentrations of background exogenous noradrenaline are summarised in Fig. 52. The results of the present protocol indicated a potentiation of the response to TNS by low background concentrations of exogenous noradrenaline. Identical results were obtained when the exogenous noradrenaline was added cumulatively and the response determined at each stage instead of discreet concentrations with rinses between as explained in the Methods.

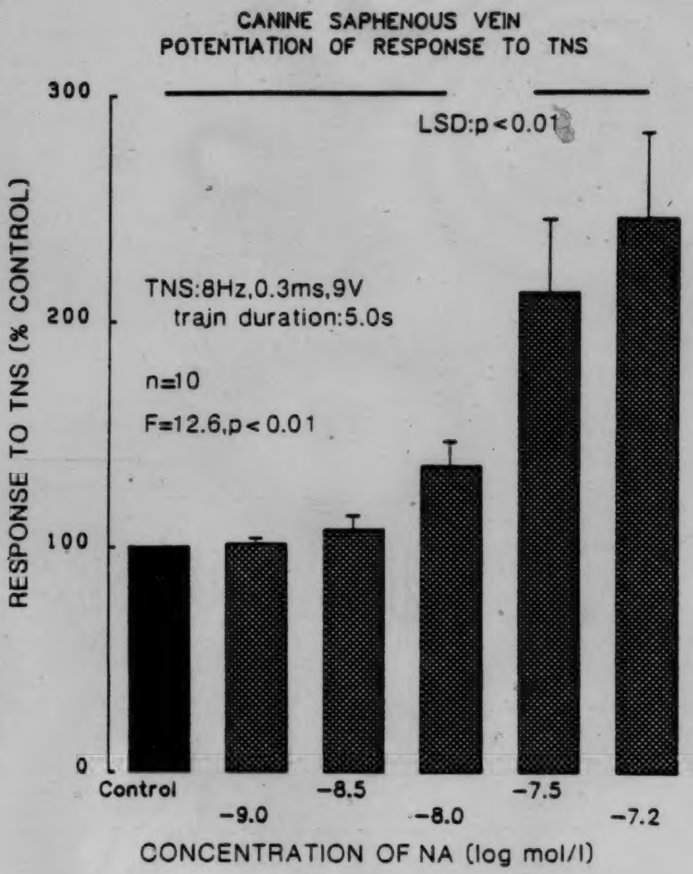

Figure 52. Summary of results from Protocol Four. A concentration dependent potentiation of the response to TNS by exogenous noradrenaline (NA) is shown. TNS:transmural nerve stimulation. LSD:least significance difference test.

DISCUSSION

The results described in the preceeding chapter can be summarised as follows:

Interaction Experiments

Protocol 1.1: The observed contraction to exogenous noradrenaline when added against a background contraction produced by TNS was significantly less than the calculated expected contraction. Further the observed contraction/expected contraction ratio had a significant negative correlation with the magnitude of the background contraction produced by TNS.

Protocol 1.2: The observed contraction to TNS against a background contraction produced by exogenous noradrenaline was, on the other hand, significantly more than the calculated expected contraction. The observed contraction/expected contraction ratio had no significant correlation with the magnitude of the background contraction produced by exogenous noradrenaline.

Protocol 1.3: The inhibition of the exogenous noradrenaline induced contraction by background TNS was not evident when this background TNS contraction was blocked by using either guanethidine or diltiazem while maintaining the electrical current.

Protocol 1.4: The additional contraction produced by exogenous nroadrenaline against a background contraction produced by either tyramine, methoxamine, histamine or phenylephrine was significantly more than the contraction produced against a background contraction of similar magnitude produced by TNS.

Protocol 1.5: The inhibition of the exogenous noradrenaline induced contraction by background TNS was not significantly decreased by the presence of either propranolol, indomethacin, aminophylline or cimetidine in the tissue bath during the protocol.

Superfusion experiments

Protocol 2.1: In this superfused saphenous vein preparation, exogenous noradrenaline was applied against a background contraction produced by TNS (as in Protocol 1.1) to examine the release of ^{3}H-noradrenaline induced by TNS. It was found that the exogenous noradrenaline significantly depressed the amount of ^{3}H-noradrenaline as well as the total radioactivity (i.e., ^{3}H-noradrenaline and its metabolites) in the superfusate.

Protocol 2.2: In this protocol TNS was applied against a background contraction produced by exogenous noradrenaline (as in Protocol 1.2). The presence of a background of exogenous noradrenaline decreased the amount of ^{3}H-noradrenaline as well as the total radioactivity present in the superfusate during TNS, as compared with that present during the application of TNS by itself.

Relaxation experiments

Protocol 3.1: Following blockade of the contractile response of saphenous vein rings to TNS (using guanethidine and phenoxybenzamine), application of TNS on a pre-contracted vein ring elicited a frequency-dependent relaxation. The response was more consistent and reproducible, and the frequency-dependency more evident with intermittent trains of stimuli (at different frequencies) as compared with continuous "cumulative" stimulation. Mechanical de-endothelialisation of the vein rings had no significant effect on the relaxatory response.

Protocol 3.2: Tetrodotoxin, cimetidine, indomethacin, aminophylline, ascorbic acid and catalase had no significant effect on the relaxatory response to TNS observed in Protocol 3.1. However, the response was almost abolished by ouabain and zero-K^+ Krebs buffer solution.

Protocol 3.3: When Protocol 3.1 was carried out following storage of the excised saphenous veins at 4°C for 9 days in Krebs buffer the relaxatory response to TNS was no longer evident.

Protocol 3.4: Chemical sympathectomy using 6-hydroxydopamine had no significant effect on the relaxatory response to TNS observed in Protocol 3.1.

Calculation of expected contraction in Protocol One

In protocol 1.1 the effect of a background TNS induced contraction, on the contractile response to exogenous noradrenaline was investigated. It was found that the observed contraction was 82.4 per cent (mean) of the expected contraction. Further, the observed contraction/expected contraction ratio showed a significant negative correlation with the magnitude of the background TNS contraction. The calculation of the expected contractions was an essential part of the analysis in this protocol for reasons outlined in the Methods section. However, both the inhibition of exogenous noradrenaline contraction by background TNS (as compared with the controls) and the significant negative correlation between the observed contraction/expected contraction ratio were present even when the analysis was carried out with the unmodified data. In fact, both these findings were much more evident with the unmodified data. Thus, calculation of the expected contractions did not qualitatively alter the results. Nevertheless, calculation of the expected contractions as outlined may not be without error.

First, the method does not take into consideration the changes in the response of the preparations with the passage of time during the experiment. This occurs as the dose-response curve carried out at the commencement of the experiment was utilised in the calculation of the expected contractions. Preliminary experiments (not reported in the thesis) demonstrated that the maximum contraction to exogenous noradrenaline decreased by less than 15 per cent over an 8-10 hour period during an experiment. However, if the maximum contraction of each dose-response is taken as a 100 per cent, the percentage change at any one concentration of noradrenaline was less than 5 per cent except at the lowest part of the dose-response curve.

A second error associated with the calculation of the expected contractions may result from equating contractions to TNS, to concentrations of exogenous noradrenaline. Although the contractile response to TNS is also dependent on noradrenaline (released at the nerve endings), the relative contribution of α_1 and α_2 receptors to the contractile response to endogenous noradrenaline (released by TNS) may be different to the relative contribution of α_1 and α_2 receptors in the case of exogenous noradrenaline (see below). Therefore equating contractions to TNS, to a concentration of exogenous noradrenaline may not be quite accurate. Although a quantitative estimate for the errors resulting from the two factors cannot be calculated, these errors are unlikely to have produced the difference in the observed contraction/expected contraction ratios between Protocol 1.1 and Protocol 1.2.

The "inhibitory" effect of background TNS on the contraction produced by exogenous noradrenaline observed in Protocol 1.1 was obtained using a wide range of concentrations of the exogenous drug and a wide range of frequencies of stimulation with TNS. Thus, the present study, unlike most other similar studies, (on the interaction between exogenous noradrenaline and TNS) provides data on this interaction spanning the whole range of the dose-respone curves rather than on a few selected points. Only the upper 10-15 per cent of the dose-response curves were excluded as it was necessary to avoid any limitation on the contraction produced during the experiment by the maximum contraction attainable with each preparation.

In Protocol 1.2 the "reciprocal of protocol 1.1" was carried out. Thus the response to TNS was measured against a background contraction produced by exogenous noradrenaline. The expected contractions were calculated to compensate for the different levels of active background tension produced by exogenous noradrenaline during the protocol (Fig. 14). This yielded an unexpected result in that the additional contraction produced by TNS against a background of exogenous noradrenaline was found to be significantly more than the expected contractions. Further, there was no significant correlation between the observed contraction/expected contraction ratio and the magnitude of the background noradrenaline contraction.

The results obtained in Protocol 1.1 where background TNS was found to inhibit the contraction produced by exogenous noradrenaline could be explained on the basis of three hypotheses.

1. Pre-synaptic α_2-inhibition(5,15) may account for the results: During Step D of Protocol 1.1 exogenous noradrenaline was added to the tissue bath against a background contraction induced by TNS. The response to the exogenous noradrenaline was measured as the

additional contraction produced at this point. This assumes that the contraction produced by TNS remained unaltered during this period. However, it is possible that the exogenous noradrenaline added may in fact depress the TNS contraction by acting on pre-synaptic α_2-receptors. This would result from an inhibition of the release of endogenous noradrenaline at the adrenergic nerve endings leading to a decreased concentration of endogenous noradrenaline at the synaptic cleft and thus a decreased contractile response. Such a pre-synaptic effect would lead to an under-estimation of the contractile response to exogenous noradrenaline during Step D and thus to the conclusion that background TNS inhibits the contraction by exogenous noradrenaline.

2. The simultaneous release of a relaxatory (vasodilator) substance by TNS may account for the results. If an inhibitory transmitter is released by TNS in addition to the endogenous noradrenaline (which produces the contractile response to TNS), this could lead to an inhibition of the contractile response to the exogenous noradrenaline when the latter is added to the tissue bath against a background of TNS. If such a relaxatory transmitter were released it could theoretically arise from two posible sites(218,219). It could arise from a separate vasodilator nerve present together with the adrenergic constrictor nerves in the blood vessel wall. As the technique of transmural nerve stimulation results in the passage of a field of current throughout the tissue, it is likely to lead to activation of any other intramural nerves present, together with the activation of

the adrenergic nerves (as long as their thresholds for activation are comparable). On the other hand, the inhibitory transmitter may exist together with noradrenaline in the adrenergic nerve ending as a co-transmitter(219,220). Adenosine triphosphate, an inhibitory transmitter in some non-adrenergic, non-cholinergic ('purinergic') nerves, is believed to be present together with noradrenaline in adrenergic nerve endings(219). There is suggestive evidence for the existence for other transmitters such as somatostatin and enkephalin as co-transmitters in adrenergic neurones(218).

3. An interaction between post-synaptic α_1 and post-synaptic α_2-receptor effects may account for the results. If, as postulated in the Introduction, endogenous noradrenaline released by TNS acts relatively more on the junctional α_1-receptors and the exogenous noradrenaline relatively more on the extra-junctional α_2-receptors, interaction between the effects of α_1- and α_2-receptor stimulation could take place. These α_1/α_2 interactions have not been investigated to date to any great extent(16). McGrath (16) in his review, suggests that such interaction between the two α-receptors sub-types could arise as a result of one or more common steps between receptor activation and the contraction of the smooth muscle. Although two articles(221,222) are cited in this review as demonstrating evidence for an interaction, the findings in these articles are not conclusive. Thus, if prior α_1-receptor stimulation (produced by TNS) were to inhibit the effects of α_2-receptor stimulation (produced by exogenous noradrenaline) findings similar to that observed in Protocol 1.1 may result.

The presence of both α_1 and α_2 post-synaptic receptors have been demonstrated in the canine saphenous vein (used in the present study) by De Mey and Vanhoutte(12,223). In this study, yohimbine (an α-receptor antagonist with a greater affinity for the α_2 sub-type) was found to cause a parallel shift to the right of the dose-response curves to noradrenaline in isolated saphenous vein rings (also in splenic artery, femoral artery and femoral vein) although the slope of the Schild plot was found to be significantly less than unity (0.69). Prazosin, a relatively selective α_1-antagonist was also found to cause a shift of the dose-respone curves to noradrenaline to the right although it was not a true parallel shift as the maximum was depressed at some concentrations. The presence of both α_1 and α_2 receptors was demonstrated by using relatively selective α_1-agonists (phenylephrine, methoxamine) as well as α_2-agonists (clonidine, tramazoline) in this study. The authors concluded that exogenous noradrenaline acted on both α_2 and α_1 post-synaptic receptors in producing contraction of the saphenous vein rings with the lower concentrations of the amines acting mainly on the α_2 receptors. Unfortunately, the effects of selective antagonists on the TNS induced contraction in the saphenous vein was not investigated in this study.

In summary, as the presence of both α_1 and α_2 post-synaptic receptors and the action of exogenous noradrenaline on both types of receptors have been demonstrated in the canine saphenous vein, an interaction between the two types of receptors remains a possible explanation for the findings in Protocol 1.1.

Influence of sympathetic nerve activity upon the sensitivity to exogenous noradrenaline

Review of the literature relating to TNS/exogenous noradrenaline interaction revealed relatively few studies. For instance, subsensitivity to exogenous noradrenaline associated with electrical stimulation was reported by Rapoport and Bevan in the isolated ear arteries of the rabbits(224).

Arterial rings taken from rabbits sacrificed by stunning followed by rapid exsanguination (a procedure accompanied by a large increase in sympathetic tone) were found to be less sensitive to noradrenaline than arteries taken from rabbits sacrificed by a lethal dose of pentobarbital (a procedure accompanied by decreased sympathetic activity) in this study. This subsensitivity lasted for at least 6 hours. Further, the response to noradrenaline (4.6 x 10^{-8} mol/l) was tested following eight 2 minute periods of TNS over a period of 2.5 hours (pulse duration 0.3 ms, frequency 16 Hz, supramaximal voltage) and compared to the response following periodic applications of histamine (2 x 10^{-6} mol/l). The response to noradrenaline following prior stimulation with TNS was significantly less than that following prior treatment with histamine. However, there was no significant difference between the former and the response to controls which were kept unchallenged during the 2.5 hour period of periodic TNS application. The authors concluded that increased sympathetic activity results in a subsensitivity of vascular smooth muscle to noradrenaline.

A similar decrease in responsiveness to sub-maximal concentrations of noradrenaline, histamine and 5-hydroxytryptamine was observed by the same authors following periodic direct electrical stimulation in rabbit ear arteries (225,226). A voltage of 15-30 V across the electrodes in the presence of tetrodotoxin to block action potentials was utilised to effect direct stimulation of the muscle. There was no difference in response to KCl (26.4 mmol/l) or the maximum contractile responses to noradrenaline, histamine or 5-hydroxytryptamine. Direct electrical stimulation failed to alter the response of rabbit saphenous veins to noradrenaline or histamine in this same study. Bevan and Rapoport concluded that the subsensitivity observed in the ear arteries was likely to be due to a change in the smooth muscle cells, beyond the receptors, as the decreased sensitivity was relatively non-specific, affecting noradrenaline, histamine and 5-hydroxytryptamine.

Unlike in Protocol 1.1 and Protocol 1.2 where the effects of a combination of TNS and exogenous nroadrenaline were investigated, the studies discussed above dealt with the effects of prior electrical stimulation (transmural and direct) on the respone to a subsequent application of exogenous noradrenaline. However, they are mentioned here as they represent the only studies in the recent literature which have shown a subsensitivity to noradrenaline produced by electrical stimulation in vascular smooth muscle. On the other hand, a supersensitivty to exogenous noradrenaline induced by continuous nerve stimulation, following pre-treatment with reserpine in the nictitating membrane of the spinal cat was shown by Trendelenberg(227). Following pre-treatment with reserpine, 3 mg/kg intraperitoneally (24 hours prior to the experiment), continuous pre-ganglionic stimulation was found to shift the dose-response curve to (ℓ)-noradrenaline by a factor of 4.2. This change in sensitivity disappeared rapidly following the termination of the nerve stimulation. Thus, this short-term pre-treatment with reserpine did not alter the sensitivity of the nicitiating membrane to noradrenaline in the unstimulated state. The findings in this study were explained on the basis of noradrenaline taken up by uptake$_1$ into the adrenergic nerve endings being immediately released during nerve stimulation (but not in the unstimulated state) leading to the supersensitivity.

In summary, a survey of the literature did not offer an adequate and satisfactory explanation for the findings in Protocol 1.1 which demonstrated an inhibition of the response to exogenous noradrenaline by background TNS.

Influence of exogenous noradrenaline upon the sensitivity to transmural nerve stimulation

The findings in Protocol 1.2 where background exogenous noradrenaline appeared to facilitate the response to TNS are even more puzzling. Hope, Law et al.(228) investigated the effects of noradrenaline and adrenaline on the vasoconstrictor response to TNS and on the efflux of ^{3}H-noradrenaline produced by TNS in the isolated rabbit ear artery(228). TNS was applied as a train of impulses at 5 Hz for 30 seconds (pulse duration 1.0 ms, supramaximal voltage) Exogenous noradrenaline was used in three concentrations: 0.05, 0.5 and 5.0 μmol/l. Superfusion with 0.5 and 5.0 μmol/l concentrations of noradrenaline resulted in a marked decrease in the additional contractile response to the train of TNS. Superfusion with noradrenaline at 0.05 μmol/l decreased the response to TNS to approximately 80 per cent of the control value. This was accompanied by decreases in the efflux of the total tritiated material (^{3}H-noradrenaline and its metabolites: no separation carried out). Similar results were obtained with adrenaline. The authors concluded that the vasoconstrictor response to TNS was decreased by exogenous noradrenaline and adrenaline as a result of pre-junctional inhibition and by other mechanisms such as the desensitisation of the post-junctional receptors. The evidence for the latter was the fact that the responses to TNS were reduced even after cessation of noradrenaline administration at a time when the transmitter release had returned to the control value or even exceeded it. As no allowance was made for the increased background active tension produced by the exogenous noradrenaline the interpretation of the decreased response to TNS during exogenous noradrenaline administration is made difficult. However, the subsensitivity to TNS following cessation of administration of noradrenaline when the tension had returned to basal levels appears more conclusive.

A study carried out by Su(229) however, demonstrated different results. The effects of low concentrations of noradrenaline, adrenaline, phenylephrine, methoxamine, naphazoline and oxymetazoline, on the vasoconstrictor response to a 40 second train of TNS (8 Hz, 0.3 ms duration) were investigated in a variety of rabbit blood vessels in this study. The α-agonists mentioned above were used in concentrations which did not cause any contractile effects on their own. The response to TNS in mesenteric arterial rings were markedly potentiated by sub-threshold concentrations of the above drugs. This effect was well maintained for at least 30 minutes and was concentration dependent. A large part of this potentiating effect developed within 5 seconds. The same concentrations of noradrenaline which potentiated the contractile response to TNS, decreased the stimulation-induced efflux of ^3H-noradrenaline in this preparation. This potentiating effect was not prevented by uptake$_1$ inhibition (cocaine), uptake$_2$ inhibition (metanephrine) or beta-blockade (propranolol). The rabbit ear artery, the saphenous artery and the pulmonary artery did not show this potentiation of TNS induced contraction by α-agonists. In fact, inhibition was produced by some agonists. The rabbit brachial artery demonstrated a slight potentiation. The authors concluded that a post-synaptic mechanism is probably involved in this potentiation (in the mesenteric artery) as the pre-synaptic actions of these agonists lead to no effect/decrease in the release of ^3H-noradrenaline in this preparation. A similar potentiation of the response of the smooth muscle to TNS was shown in the guinea-pig vas deferens by Sjostrand and Swedin(230). A number of agonists including noradrenaline, adrenaline, acetylcholine, bradykinin, histamine and substance P in concentrations that had no effects on thier own, enhanced the responses to TNS by up to 100 per cent. These drugs also enhanced the responses to direct electrical stimulation of muscle following prior (6-12 days before) surgical denervation or after blockade of action potentials in the intramural nerves with tetrodotoxin. This indicated that the enhancement was likely to be a result of a post-synaptic effect of the agonists. These studies also emphasise the need for caution against the oversimplification that a pre-synaptic inhibitory action of catecholamines would invariably hamper the transmission process as a whole. A potentiation similar to that observed in the above studies could account for the enhanced response to TNS against a background of exogenous nroadrenaline observed in Protocol 1.2.

In Protocol Four of the present study the effect of low concentrations (no contractile effects on their own) of exogenous noradrenaline on the response to a train of TNS was investigated. This demonstrated a potentiation of the response to TNS similar to that observed in the above mentioned studies. The maximum response observed amounted to 246.2 per cent of the control value. This phenomenon, if present under the conditions employed in Protocol 1.2, could account for the higher observed contractions to TNS (as compared with the expected contractions) when applied against a background contraction produced by exogenous noradrenaline. There is no reason to believe that the postulated post-synaptic potentiating mechanism would not function when the concentration of noradrenaline used produced a contraction on its own (i.e. above threshold). However, an increase in tension produced by the background noradrenaline would tend to mask the potentiation because of the added confounding factor of a decreased response to a fixed stimulus as one proceeds up the dose-response curve.

The findings in Protocol Four demonstrated also, that pre-synaptic α_2-inhibition of TNS by exogenous noradrenaline is unlikely to be of any biological significance during conditions similar to those employed in this protocol in the canine saphenous vein. The mechanism responsible for the potentiation of the response to TNS is not clear at present.

Influence of electrical current on the response to exogenous noradrenaline

In Protocol 1.3, it was found that inhibition of the exogenous noradrenaline contraction by TNS (observed in Protocol 1.1) was not evident, when the response to exogenous noradrenaline was measured following blockade of the background TNS induced contraction with guanethidine, while maintaining the electrical current. Guanethidine, an adrenergic neurone blocking agent, depletes the stores of noradrenaline from adrenergic nerve endings and also prevents the release of the transmitter. However, it has no effect on the passage of the pulses of current between the two electrodes in the tissue when 'TNS' is applied. Thus, in this protocol, what is measured is the effect of background pulses of electrical current (strength 10 V, duration 1.0 ms, different frequencies) on the response to exogenous noradrenaline. No significant effect was demonstrated. As stated before, guanethidine was used in this protocol at the lowest concentration that blocked the effect of TNS at the frequency used in the first part of the protocol, i.e., where the effect of exogenous noradrenaline was measured against a background contraction by TNS. This protocol demonstrated that the inhibition of the exogenous noradrenaline contraction was not due to the field of current per se, acting directly on the vascular smooth muscle or by causing oxidation of the exogenous noradrenaline in the tissue bath. This finding was confirmed in the experiments using the calcium channel antagonist diltiazem in place of guanethidine to block the contractile effects of TNS. An interesting observation during the latter experiments was that diltiazem in a concentration of 10^{-5} to 10^{-4} mol/l was found to block the contractile effects of TNS (at 1-4 Hz) completely while leaving the magnitude of the contraction produced by exogenous noradrenaline at about 70-80 per cent of its control value. This differential effect of Ca^{2+} channel antagonists on the contraction produced by TNS as compared with that produced by exogenous noradrenaline in the canine saphenous vein was also shown by Vanhoutte and Rimele(231) about the same time as the present study. This was demonstrated with both diltiazem and verapamil in the above study.

In Protocol 1.4 it was demonstrated that the additional contraction produced by exogenous noradrenaline against a background of TNS was significantly less than that produced against a background contraction of similar magnitude produced by tyramine, phenylephrine, methoxamine or histamine. Thus, the inhibition of the exogenous noradrenaline contraction produced by background TNS (Protocol 1.1) was not present with background contractions produced by the above named agonists. In fact, a potentiation of the exogenous noradrenaline contraction was present in the case of tyramine, methoxamine and histamine where the mean magnitude of the contractions in the presence of background agonist contractions were over 80 per cent of the control contractions by noradrenaline alone with some values even exceeding the controls..

Contraction of smooth muscle by tyramine is mediated by two pharmacological actions: (1) an indirect sympathomimetic effect which leads to the release of endogenous noradrenaline from the adrenergic nerve endings present in the tissue (2) a direct effect on smooth muscle leading to contraction. The indirect sympathomimetic effect is usually seen at lower concentrations of tyramine as compared with the latter effect. In exerting this indirect effect, tyramine is first taken up into the adrenergic nerve endings by uptake$_1$. This tyramine then leads to the displacement of endogenous noradrenaline within the synaptic vesicles. Some of the noradrenaline displaced into the neuroplasm comes out of the nerve endings and produces the contraction of the smooth muscle while the balance is metabolised by intraneuronal enzymes. In the present study, the contractile effects of the concentrations of tyramine used ($<6 \times 10^{-5}$ mol/l) were completely blocked by the uptake$_1$

inhibitor cocaine (this was established in preliminary experiments - see Results). Thus, the effects would have been produced predominantly by its indirect effects via the release of endogenous noradrenaline. Nevertheless, the inhibitory effect on the exogenous noradrenaline produced by TNS (which also releases endogenous noradrenaline) was not observed with tyramine. This could be explained in three ways.

Firstly, although both tyramine and TNS lead to the release of endogenous noradrenaline, the two mechanisms of release are not identical. Tyramine, as explained above, leads to the displacement of endogenous noradrenaline from the storage vesicles and its subsequent "leakage" into the synaptic cleft. TNS, on the other hand, leads to the release of endogenous noradrenaline via exocytosis of the storage vesicles. Here noradrenaline is released into the synaptic cleft together with other vesicular contents such as dopamine-β-hydroxylase and ATP. If the inhibitory effect of background TNS, on the exogenous noradrenaline contraction observed in Protocol 1.1 was due to the simultaneous release of an inhibitory co-transmitter, this inhibition would not be observed with tyramine which releases endogenous noradrenaline by displacement. Secondly, if the inhibitory effect observed in Protocol 1.1 was due to the release of an inhibitory transmitter released from a non-adrenergic nerve (present in the vessel wall together with adrenergic nerves) by TNS, this release would not take place in the case of tyramine whose actions on the adrenergic nerve endings depends on a specific uptake process. Thus, it is not likely to act on a non-adrenergic nerve. Thirdly, the results could be explained on the basis of competition between exogenous noradrenaline and tyramine for uptake$_1$, when noradrenaline is added against a background of tyramine(148,232). As tyramine has a higher affinity for uptake$_1$ than noradrenaline it would act as a competitive inhibitor of uptake$_1$ for noradrenaline. This would lead to a higher biophase concentration of noradrenaline and therefore a potentiation of its effects. Although this third possibility is known to occur and is likely to play some part in the results observed with tyramine, the other two possibilities cannot be excluded on the findings presented to this stage..

α_1/α_2 receptor interaction

An interaction between the effects of $\alpha_1$receptors and α_2 receptors was considered as a probable explanation for the inhibition of exogenous noradrenaline contraction by background TNS. This postulated interaction should still occur in the tyramine experiments where the endogenous noradrenaline released at the adrenergic nerve endings by tyramine would act relatively more on the intrasynaptic (probably α_1) receptors just as in the case with TNS. However, the inhibitory effect was not evident under these circumstances. Thus, the tyramine experiments do not support α_1/α_2 receptor interaction as a probable explanation for the findings in Protocol 1.1. Further evidence in favour of the above conclusion is provided by the experiments using phenylephrine and methoxamine. Both these drugs have been considered as relatively specific α_1-adrenoceptor agonists(16,233) with phenylephrine possesing a minor indirect sympathomimetic action in addition. In these experiments the effect of α_1-adrenoceptor stimulation on the contraction of exogenous noradrenaline was tested. No inhibition of the contraction by exogenous noradrenaline was evident under these circumstances. Thus an interaction between α_1 and α_2 receptor stimulation appears to be an unlikely explantation for the inhibitory effect on exogenous noradrenaline contraction by TNS in Protocol 1.1.

The experiments using methoxamine help shed further light on the potentiation observed with tyramine. Methoxamine is an extremely poor substrate for the uptake$_1$ process(153). Thus, unlike tyramine, it would not compete with the exogenous noradrenaline for the uptake$_1$. Nevertheless, the inhibition of exogenous noradrenaline contraction was not observed against a background contraction produced by methoxamine where the results were very similar to those observed with background tyramine. Thus, competition for uptake$_1$ between tyramine and exogenous noradrenaline may not be the sole explanation for the lack of inhibition of the exogenous noradrenaline contraction by a background contraction produced by tyramine.

The experimental findings up to this point leave two possibilities as explanations for the findings in Protocol 1.1. Firstly, pre-synaptic α_2-receptor inhibition by the exogenous noradrenaline remains a possibility. None of the experiments in Protocol 1.4 (with tyramine, phenylephrine, methoxamine and histamine) are against the pre-synaptic inhibition hypothesis. In fact, the experiments using tyramine may render some support to the above hypothesis. As mentioned before, the release of endogenous noradrenaline by TNS occurs by exocytosis while that induced by tyramine occurs by displacement. Although the release of noradrenaline by exocytosis is subject to pre-synaptic α_2-inhibitory control, the release produced by displacement is not(129). Exogenous noradrenaline has been shown to inhibit the release of ^{3}H-noradrenaline (in the presence of the uptake$_1$ inhibitor cocaine) produced by both TNS and K^+ depolarization in the canine saphenous vein(174). However, exogenous noradrenaline did not produce any inhibition of the release of ^{3}H-noradrenaline produced by tyramine in the above study. In Protocol 1.4 the inhibition of exogenous noradrenaline contraction by background TNS was not observed with a background of tyramine. This may have been due to the absence of pre-synaptic α_2-inhibition under the latter circumstances.

The second possibile explanation for the findings in Protocol 1.1 is simultaneous release of an inhibitory transmitter. This may appear as a co-transmitter or from a different intramural nerve. The experiments with guanethidine which blocks the release from adrenergic nerve endings are more in favour of a co-transmitter than a separate nerve. This is because no inhibition was observed under these circumstances. As guanethidine is not likely to prevent release of transmitter from a non-adrenergic nerve, the inhibition should have been still evident if the inhibitory transmitter was released from such a nerve. The experiments using diltiazem appear to support the pre-synaptic hypothesis rather than the inhibitory transmitter hypothesis. As diltiazem blocks the contractile effects of TNS and presumably not the release of noradrenaline, the inhibitory effects of a released second transmitter should have been evident under these circumstances: but no inhibition of the exogenous noradrenaline contraction was observed. However, it is known that Ca^{2+} antagonists may block the inward flux of Ca^{2+} ions at the nerve endings which trigger the release of the neurotransmitters(137). Although this usually occurs at higher concentrations than that usually necessary to block the smooth muscle membrane Ca^{2+} channels, such an action at the neuronal membrane preventing the release of the postulated inhibitory transmitter cannot be excluded.

Investigation of the possible mediators of the inhibition observed in Protocol 1.1

An attempt was made to identify the postulated relaxatory substance in Protocol 1.5 by using pharmacological antagonists against possible relaxatory mediators. Firstly, a beta-receptor mediated relaxation by the noradrenaline released by TNS was investigated by repeating Protocol 1.1 in the presence of propranalol. The presence of β-receptors in the smooth muscle of the canine saphenous veins was shown by Guimaraes et al(234,235,236). Further, noradrenaline was shown to be effective as an agonist at these β-receptors, although much weaker than both adrenaline and isoprenaline. In these investigations propranolol was used in a concentration of 5×10^{-7} to block the β-receptor mediated effects. In Protocol 1.5 of the present study propranolol used in a concentration of 10^{-5} mol/l did not abolish the inhibition of the exogenous noradrenaline mediated contraction by background TNS. In fact, the exogenous noradrenaline mediated contraction against a background of TNS was significantly less (thus the inhibition more) in the presence of propranalol. Thus, a β-receptor mediated effect is unlikely to account for the findings in Protocol 1.1.

Histamine has been shown to produce relaxation in vascular smooth muscle by acting on histamine-receptors present in the muscle cell membrane(237,238). With respect to vascular smooth muscle, histamine is found in abundance in the blood vessel wall in a non mast cell pool(237,239). Histamine is also found concentrated in autonomic nerves, especially the post-ganglionic sympathetic fibres(237,240). Thus, the possible mediation by a H_2-receptor, of the inhibition of the exogenous noradrenaline contraction by TNS was investigated by using the

H_2-receptor antagonist cimetidine. The presence of cimetidine did not significantly alter the response to exogenous noradrenaline against a background of TNS. Therefore, it is unlikely that the inhibition of exogenous noradrenaline contraction observed in Protocol 1.1 is mediated by a H_2-receptor.

During the last decade the existence of purinergic nerves in non-vascular as well as vascular smooth muscle has been documented(72,241). Adenosine triphosphate (ATP) and/or adenosine are believed to be the neurotransmitters in these purinergic nerves. Purinoceptors are of two types. P_1-purinoceptors are most sensitive to adenosine and progressively less sensitive to adenosine monophosphate (AMP), adenosine diphosphate (ADP) and ATP. On the other hand, the P_2-purinoceptors are most sensitive to ATP and progressively less sensitive to ADP, AMP and adenosine. Both ATP and adenosine have been shown to cause relaxation in canine saphenous veins pre contracted with noradrenaline(211). Further, ATP is known to be present in adrenergic nerve endings together with noradrenaline in the synaptic vesicles(130). Thus, it is possible that the inhibitory effect on the exogenous noradrenaline contraction by TNS was mediated by a purinergic substance. Aminophylline was used as a P_1-purinoceptor antagonist in the present study. The inhibition of the exogenous noradrenaline contraction by TNS was still evident under these circumstances. However, the effects of a P_2-purinoceptor antagonist was not investigated in the present study. Thus, the role for a purinergic substance as a possible mediator for the inhibitory effect on the exogenous noradrenaline contraction, cannot be ruled out with the available evidence in the present study.

Prostaglandins synthesised from arachidonic acid via the cyclo-oxygenase pathway are present in most mammalian tissues, although they are not stored to any significant extent except in the seminal fluid(242). Prostaglandins are believed to be primarily local or tissue hormones that have their effects, at or near to the site of synthesis. They exert a vasodepressor or a vasoconstrictor action on vascular smooth muscle depending on the particular prostaglandin involved, the type of blood vessel and the species tested(243). Prostaglandin-E_1 (PGE_1) has been shown to reduce the pressor response to intravenous catecholamines in the rabbit and rat vasculature(194). Further, adrenergic nerve stimulation induces synthesis and release of prostaglandins in many tissues(244). Such an effect could theoretically occur during the application of TNS in Protocol 1.1. resulting in the synthesis and release of an inhibitory prostaglandin. Thus, the effect of the cyclo-oxygenase inhibitor indomethacin on the response to exogenous noradrenaline against a background of TNS was tested in the present study. As the inhibitory effect of TNS on the exogenous noradrenaline was still evident under these circumstances an arachidonic acid metabolite of the cyclo-oxygenase pathway is unlikely to be the mediator for this inhibitory effect. However, the participation of a metabolite of the lipoxygenase pathway of arachidonic acid metabolism cannot be excluded as indomethacin does not inhibit the enzyme lipoxygenase.

In summary, the experiments done in Protocol 1.5 were not fruitful in the identification of the postulated inhibitory transmitter. However, the results observed suggested that the inhibitory effect was unlikely to be mediated by a β-receptor, a H_2-receptor or a prostaglandin metabolite of the cyclo-oxygenase pathway.

Role of pre-synaptic α_2-inhibition in the findings in Protocol 1.1

In Protocol Two the role of pre-synaptic α_2-receptor mediated inhibition during the experimental conditions of Protocols 1.1 and 1.2 were investigated using ^{3}H-noradrenaline. The experiments were carried out in the presence of the uptake$_1$ inhibitor cocaine hydrochloride. In Protocol 2.1 exogenous noradrenaline added against a background TNS contraction, significantly decreased the total radioactivity as well as the intact ^{3}H-noradrenaline fraction in the superfusate. Total radioactivity was decreased to 55.8 per cent (mean) of the value during the application of TNS only (Period II and Period IV of the protocol), and ^{3}H-noradrenaline to 30.0 per cent (mean). In Protocol 2.2, the total radioactivity and the ^{3}H-noradrenaline in the superfusate during the period where TNS was applied against a background of exogenous noradrenaline was significantly less than that during the periods of application of TNS only. The total radioactivity was 73.0 per cent (mean) of its value during TNS alone and ^{3}H-noradrenaline 66.0 per cent of its value.

If it is assumed (as is usually done) that the tritiated noradrenaline in the superfusate is a satisfactory measure of the amount of noradrenaline released at the adrenergic nerve ending, there is evidence for pre-synaptic inhibition by the exogenous noradrenaline added during Protocol 2.1 and Protocol 2.2 (which were similar to Protocol 1.1 and Protocol 1.2 respectively). Further, the degree of inhibition appears to be greater in Protocol 2.1 (and thus presumably in Protocol 1.1), with the decrease in total radioactivity and the ^{3}H-noradrenaline amounting to 44.2 per cent (100-55.8) and 70 per cent (100-30) respectively as compared with 27 per cent (100-73) and 34.0 per cent (100-66) respectively in Protocol 2.2 (and thus presumably in Protocol 1.2). The TNS induced contraction was smaller in magnitude than the exogenous noradrenaline contraction in Protocol 2.1 and vice versa in Protocol 2.2. Thus, TNS was applied at a higher frequency in Protocol 2.2. The degree of pre-synaptic inhibition is known to be inversely related to the frequency of stimulation of the adrenergic nerves(129,186). Thus the difference in the degree of inhibition observed in the two protocols is not unexpected. Protocol 2.2 which utilised a higher frequency of stimulation demonstrated a lower degree of inhibition as expected.

Superficially this difference in the degree of inhibition may seem to account for the different results observed in Protocol 1.1 and Protocol 1.2. In Protocol 1.1 background TNS was found to inhibit the contraction by exogenous noradrenaline. In Protocol 1.2 a background exogenous noradrenaline was found to enhance the contraction by TNS. However, Protocol 2.1 and Protocol 2.2 were carried out only at a single frequency (in each case) of TNS and a single concentration of exogenous noradrenaline. This tended to highlight the difference in the degree of inhibition in the two protocols. Protocol 1.1 and Protocol 1.2 , on the other hand, were carried out using a multitude of concentrations of exogenous noradrenaline and a multitude of frequencies of TNS. The difference between the two protocols was evident throughout the range of TNS employed (refer Fig. 24 and Fig. 27). This makes it unlikely that the difference in the degree of pre-synaptic inhibition would account for the difference observed between Protocol 1.1 and Protocol 1.2.

Inhibition of the TNS induced release of ^{3}H-noradrenaline by exogenous noradrenaline in the canine saphenous vein (similar to that observed in the Protocol 2.1 and Protocol 2.2 of the present study), has been demonstrated by Lorenz et al(174). In this study the effect of exogenous noradrenaline on the release of tritiated material produced by TNS was investigated also in the absence of an uptake$_1$ inhibitor. The addition of noradrenaline (1.2×10^{-6} mol/l) to the superfusing fluid increased the tension (by 3.7 g mean ± 0.6 g SEM), the total radioactivity of the superfusate, the efflux of ^{3}H-noradrenaline, its deaminated metabolites and normetanephrine. When this same concentration of ^{3}H-noradrenaline was added against a background contraction by TNS (2 Hz, 9 V, 2.0 ms) the further increase in tension was minimal (<0.2 g) (Fig. 53). This was similar to the results observed in Protocol 1.1 of the present study. Further, with the addition of exogenous noradrenaline in the above study, there was no decrease in the total radioactivity and the ^{3}H-noradrenaline in the superfusate. The only significant effect was an augmentation of the efflux of deaminated metabolites of ^{3}H-noradrenaline. Thus, in the absence of uptake$_1$ inhibition (the condition present in Protocol 1.1 and Protocol 1.2 of the present study), pre-synaptic inhibition by exogenous noradrenaline could not be demonstrated in the canine saphenous vein. Investigations using canine abdominal aorta, superior mesenteric artery, splenic artery and the splenic capsule too showed a similar inability to demonstrate pre-synaptic inhibition in the absence of uptake$_1$ blockade in the above study. Only the canine portal vein showed an inhibition of the efflux of ^{3}H-noradrenaline by exogenous noradrenaline in the absence of cocaine.

The results of the above study were explained on the basis of a possible displacement by exogenous noradrenaline, of the ^{3}H-

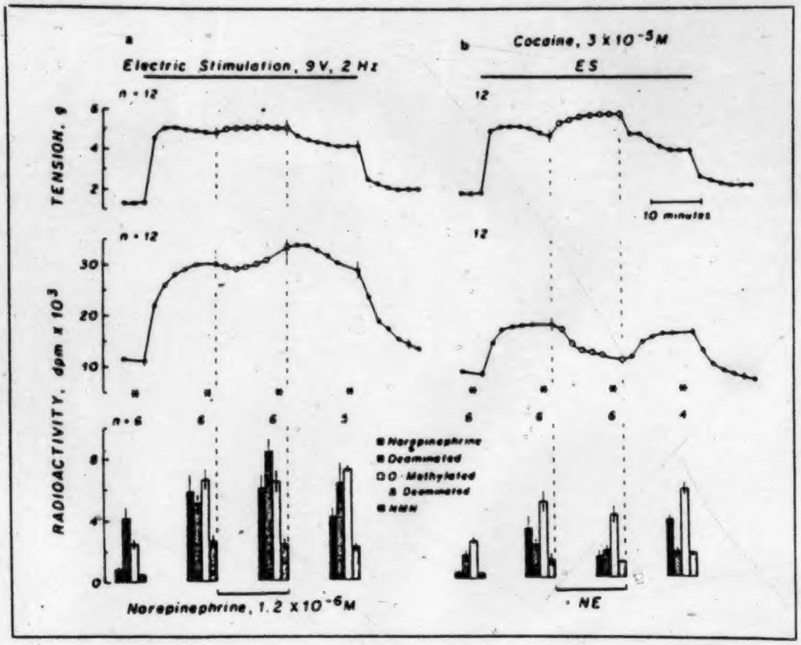

Figure 53. Effect of noradrenaline on tension, total radioactivity of superfusate and efflux of ^{3}H-noradrenaline and metabolites during electrical stimulation of canine saphenous vein strips before (a) and after (b) cocaine (3 x 10^{-5} mol/1). The asterisks indicate times at which superfusate was analysed chromatographically to determine levels of ^{3}H-noradrenaline and metabolites. Values shown are mean ± SEM (Reproduced with permission from S Karger AG; Basel, Blood Vessels 1979; 16:113-125).

noradrenaline within the adrenergic nerve terminal obscuring the demonstration of the pre-synaptic inhibition(174). This was supported by the observation that during TNS, exogenous noradrenaline augmented the efflux of deaminated metabolites in the absence of cocaine but not in its presence. As mentioned before, exogenous noradrenaline (by itself) also produced an increase in the efflux of total radioactivity as well as ^{3}H-noradrenaline. The latter finding makes it difficult to draw a firm conclusion about the pre-synaptic effects of exogenous NA during TNS. However, whether the noradrenaline comes out of the adrenergic nerve terminal by displacement or exocytosis would not make a difference to its contractile effects as long as it comes out in the form intact (unmetabolised) noradrenaline. Thus, tyramine, which acts predominantly by causing displacement of noradrenaline from adrenergic nerve terminals, is still able to cause a powerful contractile effect in vascular smooth muscle. In the above study, although the added exogenous noradrenaline may have produced an increased efflux of deaminated metabolites the amount of unmetabolised ^{3}H-noradrenaline in the superfusate remained unchanged (Fig. 51). Thus, the contraction produced by TNS should have remained unchanged during the period of addition of exogenous noradrenaline. In spite of this the additional contraction produced by exogenous noradrenaline was less than 0.2 g as mentioned before.

In summary, no evidence for pre-synaptic inhibition by exogenous noradrenaline was available in the canine saphenous vein in the absence of uptake$_1$ inhibition although one cannot definitely exclude such a phenomenon in this experimental situation. This finding makes it difficult to conclude that pre-synaptic α_2-inhibition is the mechanism responsible for the inhibitory effect of TNS, upon the contraction produced by exogenous noradrenaline observed in Protocol 1.1. Another observation also argues against the pre-synaptic inhibition mechanism. Pre-synaptic inhibition is known to decrease with increasing frequency of stimulation(129,186). Thus, if pre-synaptic inhibition were to account for inhibition of the contraction of exogenous noradrenaline in Protocol 1.1, one would expect this latter inhibition to be less with increasing frequency of TNS. However, the observed/expected contraction ratio showed a significant tendency to decrease with increasing magnitude of background TNS in Protocol 1.1 (Fig. 24). The observed/expected contraction ratio had a significant negative correlation (-0.42) with the magnitude of the background TNS. Although this correlation coefficient is relatively low, it is emphasised that one would expect a positive correlation coefficient if pre-synaptic inhibition were to account for the findings in Protocol 1.1.

Relaxation to TNS

As pre-synaptic α_2 inhibition did not seem likely to account for the inhibition of exogenous noradrenaline contraction by TNS in Protocol 1.1, another possibility that may account for this finding, the concurrent release of an inhibitory neurotransmitter by TNS had to be considered. This was investigated in Protocol 3.1 following blockade of the contractile response to TNS. The adrenergic neurone blocker guanethidine (10^{-4} mol/l) and the α-antagonist phenoxybenzamine had to be used in combination to effect a blockade of the contractile response to TNS up to a frequency of 32 Hz. Although both the above drugs were used in relatively high concentrations, this was unavoidable as the canine saphenous vein is extremely responsive to TNS with a maximum contraction of about 90 per cent of the maximum contraction to exogenous noradrenaline. The vein rings pre-contracted with prostaglandin $F_{2\alpha}$ following this blockade demonstrated a frequency dependent relaxation to TNS applied as intermittent trains of stimuli. As the experiments were carried out in the presence of both atropine and propranolol, the relaxation could be described as non-adrenergic, non-cholinergic in nature. The relaxatory response demonstrated a slow recovery and a biphasic response especially at the higher frequencies.

Relaxatory responses to TNS, similar to those observed in the present study, have been demonstrated in certain cerebral arteries(245,246,247) and canine coronary arteries in vitro(248). For instance, Bevan et al(246) described a neurally mediated vasodilatation in cat cerebral and extra-cerebral arteries. The relaxation observed was biphasic in nature with an atropine (5×10^{-7} mol/l) sensitive component followed by a delayed atropine resistant component. In the present investigation, although a biphasic response was observed occasionally (especially at the higher frequencies of stimulation), both components of the response were found to be resistant to atropine at a concentration of 5×10^{-6} mol/l. Ito and Takeda(212) described a relaxatory response to TNS in the cat tracheal smooth muscle following an increase of tone produced by 5-hydroxytryptamine. This relaxatory response was reduced by propranolol (2×10^{-6} mol/l) but a significant part of the response was non-adrenergic and non-cholinergic in nature. The authors remarked on a slow recovery from the relaxation produced by the transmural nerve stimulation, similar to that observed in the present study.

Mechanism responsible for the relaxatory response to TNS

TNS leads to contraction or relaxation in smooth muscle by the activation of intramural nerves. This is achieved by induction of action potentials in these nerves with a resultant release of neurotransmitter from the nerve endings. Tetrodotoxin, a specific fast Na^+ channel blocker has been used as a test for the neural origin of relaxatory or contractile responses to TNS in isolated smooth muscle(249), since it is believed to block the action potentials in the intramural nerves. This phenomenon was investigated in Protocol 3.2. Tetrodotoxin, in a concentration of 10^{-6}mol/l, did not significantly affect the relaxatory response to TNS observed in Protocol 3.1. However, this same concentration of tetrodotoxin almost completely abolished the contractile response to TNS (up to a frequency of 32 Hz), prior to guanethidine and phenoxybenzamine treatment in the present study. In most of the studies cited above(245,246,247) tetrodotoxin abolished the relaxatory responses to TNS. Thus, the inability to block the relaxation by tetrodotoxin in the present study could be considered as evidence against a neural origin for this relaxation. However, recent evidence indicates the existence of tetrodotoxin resistant nerve action potentials and neurotransmitter release in mammalian nerves(250,251,252). Hardebo et al(253) also have described a relaxatory response to TNS which was resistant to tetrodotoxin in isolated rabbit and cat, cerebral and coronary arteries. Thus it could be argued that these effects are mediated by, as yet unidentified, tetrodotoxin resistant intramural nerves.

Another test which has been employed often to establish the neural origin of responses, is the examination of the response to TNS following storage of isolated tissue at 4°C in a buffer solution for 9-10 days(254). Prolonged cold storage has been shown to cause slow degeneration of the intramural nerves with irreversible loss of ability to store and release neurotransmitter in isolated smooth muscle(15,213). In Protocol 3.3 of the present study after a period of nine days in storage at 4°C, TNS produced no contractile responses in the saphenous veins, indicating a "denervation" of the sympathetic nerves. However, these preparations responded to prostaglandin $F_{2\alpha}$ and to isoprenaline in the conventional manner with contraction and relaxation respectively although the contractile response was significantly less than the controls. In such veins the relaxatory responses to TNS were abolished also. These findings indicate that a neural mechanism may indeed be involved in the observed relaxatory response.

A second potential mechanism which may mediate this relaxatory response is a direct (non-neurogenic) effect on the smooth muscle cells by the electrical impulses per se. Such an explanation was offered by Rooke et al(248) for the relaxatory response to transmural nerve stimulation observed in the canine coronary arteries since this response was resistant to prolonged cold storage as well as to tetrodotoxin. Although the abolition of the relaxatory response following cold storage in the present study suggests a neurally mediated response, it does not exclude a direct relaxatory effect on the smooth muscle because cold storage also could have had a specific effect upon a hypothetical mechanism responsible for such a direct relaxatory effect.

Site of origin of the postulated inhibitory neurotransmitter

If an inhibitory neurotransmitter released by the TNS was responsible for the relaxation observed in Protocol 3.1, it could theoretically originate from two sites (1) from an as yet, unidentified inhibitory intramural nerve (2) from the synaptic vesicles of adrenergic nerves where it may exist as a co-transmitter together with noradrenaline. The latter possibility does not seem likely as the relaxation to TNS in Protocol 3.1 was demonstrated following guanethidine treatment which would block the release of noradrenaline from sympathetic nerves. Nevertheless, this was further investigated by using 6-hydroxydopamine to produce a chemical sympathectomy in the venous rings. 6-hydroxydopamine treatment results in degenerative changes in adrenergic nerves such as cytoplasmic shrinking, virtual absence of dense-core vesicles and mitochondrial swelling as demonstrated by Aprigliano et al in the portal vein and caudal artery of the rat(215,255). These changes occured within 2-4 hours of the treatment with the drug. It is unlikely that a possible co-transmitter present together with noradrenaline in the dense-core vesicles would be released during TNS following pre-treatment with 6-hydroxydopamine. However, this treatment would be unlikely to affect a non-adrenergic intramural nerve as 6-hydroxydopamine exerts its effect only after it is taken up and concentrated in dense-core vesicles of adrenergic nerves by specific uptake processes (uptake$_1$ and vesicular uptake - refer to sub-section on Transmitter disposition and termination of its effects)(256). In Protocol 3.4 no significant difference in the relaxatory response to TNS was observed in the 6-hydroxydopamine treated venous rings as compared with control rings. Thus it appears unlikely that an inhibitory co-transmitter present together with noradrenaline in adrenergic nerves is responsible for the relaxatory response to TNS observed in the present study. Further support for this conclusion is provided by the inability of tetrodotoxin to significantly affect the relaxatory response to TNS in Protocol 3.2. As tetrodotoxin is known to block the release of noradrenaline from adrenergic nerve endings (by blocking the fast Na^+ channel of the action potential) it would also block the simultaneous release of a co-transmitter.

Possible mediators of the relaxatory response to TNS

Free radicals have been implicated as a possible mediator of relaxatory responses in vascular smooth muscle(257). A free radical is defined as any atom, group of atoms, or molecule in a particular state with one unpaired electron occupying an outer orbital ("biradical" - two unpaired electrons)(258). These radicals have been implicated in a variety of disease states in recent years(258) and have also been shown to produce relaxation in some blood vessels(259). Hyperoxia is one of several factors that are believed to precipitate the generation of free radicals in living tissue(258). In isolated smooth muscle experiments the tissue bath is oxygenated with a mixture of 95 per cent O_2 and 5 per cent CO_2. This mixture has been shown to produce O_2 tensions of around 640 mm Hg in the tissue bath solution(260). The passage of pulses of current through such a hyperoxic medium (during TNS) could be regarded as a potential trigger for the generation of these free radicals. Thus, free radicals can be considered as a potential mediator of the relaxatory phenomenon observed in Protocol 3.1 of the present study. The possible role for these radicals are generally investigated by using drugs that function as "free radical scavengers"(259). However, this is made difficult by the existence of a variety of free radicals and a variety of specific as well as non-specific scavengers(258). In

Protocol 3.2 of the present study the effect of two such scavengers on the relaxatory response to TNS was investigated. The two drugs used were catalase (a scavenger of hydrogen peroxide and peroxide radicals) and ascorbic acid (a non-specific scavenger)(258,259). These two drugs did not significantly alter the relaxatory response to TNS. Absence of an effect by catalase suggests that hydrogen peroxide is unlikely to play a role in the relaxation observed in the present study. The conclusion to be drawn from the lack of effect of ascorbic acid is more difficult.

In addition in Protocol 3.2 the effect of cimetidine (H_2-receptor antagonist), indomethacin (cyclo-oxygenase inhibitor), aminophylline (P_1-purinoceptor antagonist) on the relaxatory response to TNS was investigated. None of these drugs had a significant effect on the response to TNS. Thus, it is unlikely that the relaxation to TNS observed in Protocol 3.1 is mediated by a H_2-receptor stimulation, a P_1-purinoceptor stimulation or by an arachidonic acid metabolite of the cyclo-oxygenase pathway.

The Na^+/K^+ ATPase inhibitor ouabain abolished the relaxatory responses observed in the present study. Since a relatively high concentration of the drug (2×10^{-4} mol/l) was necessary for this blockade a non-specific effect of ouabain unrelated to the membrane Na^+/K^+ ATPase inhibition has to be considered. However, zero-K^+ Krebs buffer solution which inhibits the Na^+/K^+ pump, also abolished the relaxatory response to TNS. Thus it appears likely that the relaxatory response is dependent on a functional Na^+/K^+ pump.

Influence of mode of TNS on the relaxation

An interesting additional observation was the fact that intermittent transmural nerve stimulation produced a greater and more consistent relaxation than continuous stimulation of the isolated venous ring. This is in contrast to the sympathetically mediated contractile effects of transmural nerve stimulation in the isolated canine saphenous vein where continuous stimulation produces a consistent, frequency dependent response(261). Although a frequency dependent uniform response to continuous transmural nerve stimulation is usual in the classical autonomic nerves, a significantly enhanced response to intermittent bursts of stimulation has been described by Edwards and Bloom in the gastro-intestinal tracts of calves(262). This response was associated with the release of a bombesin-like-peptide from intramural nerves from the gastro-intestinal tract. The unusual finding with intermittent transmural nerve stimulation in the present study could be explained also by an enhanced release of such a neurotransmitter.

In summary, the findings of Protocol Three supported the hypothesis that TNS produces a non-adrenergic, non-cholinergic relaxation in the canine saphenous vein pre-contracted with prostaglandin $F_2\alpha$. This relaxation, although resistant to tetrodotoxin, was abolished by cold storage of the veins for 9 days. It is therefore suggested that this relaxatory response could be mediated by an as yet unidentified, tetrodotoxin resistent relaxatory nerve although a direct effect of TNS on the smooth muscle cannot be definitely excluded. Even if the latter mechanism were responsible for the relaxation, it would still be potentially important in isolated smooth muscle experiments as it is likely to occur during TNS in such experiments.

Role of TNS induced relaxation in Protocol 1.1

Finally, one has to consider the role played by this TNS induced relaxation (in Protocol 3.1), in the inhibition of the exogenous noradrenaline contraction by background TNS observed in Protocol 1.1. Although TNS induced relaxation in Protocol 3.1 was demonstrated following the blockade of the contractile response, there is no reason to believe that the same phenomenon would not occur in the absence of such blockade. However, the relaxatory response would not be apparent because of the bigger magnitude of the contractile effect. Thus, this TNS induced relaxation could theoretically account for the inhibition of the exogenous noradreanline contraction by background TNS observed in Protocol 1.1. Both effects were not antagonised by propranolol, cimetidine, aminophylline, or indomethacin. The inhibitory effect observed in Protocol 1.1 was present with a background contraction with TNS only; other agonists substituted in place of TNS failed to produce any inhibition. Tyramine, which produces its contractile effects by releasing endogenous noradrenaline too did not produce inhibition indicating that the inhibitory effect was dependent on TNS rather than on endogenous noradrenaline. In addition, the inhibitory effect observed in Protocol 1.1 was found to be significantly more (or the observed/expected contraction ratio less) with increasing magnitude of background TNS. This relationship, as mentioned before, is against presynaptic inhibition being the mechanism responsible for the findings of Protocol 1.1. However, the above relationship could be explained on the basis of the findings of Protocol 3.1 where the magnitude of the relaxatory effect was dependent on the frequency of TNS.

Although the above evidence is suggestive, some facts remain unexplained at the present time. (1) The findings in Protocol 1.2 where a background exogenous noradrenaline contraction was found to potentiate the contraction by TNS, is not readily explainable on the above hypothesis. The "relaxatory effect" produced by the TNS when applied against a background of exogenous noradrenaline should have limited the total contraction. (2) The inhibitory effect on exogenous noradrenaline contraction was not observed in Protocol 1.3 where the contraction produced by TNS was blocked using guanethidine or diltiazem while maintaining the electrical current. Why the relaxatory effect to TNS observed in Protocol 3.1 did not limit the contraction by exogenous noradrenaline is not clear.

These discrepancies may be explainable on the basis of a number of confounding mechanisms operating during Protocol 1.1 and Protocol 1.2 where the interaction between exogenous noradrenaline and TNS was studied. (1) The possible effects of pre-synaptic α_2-inhibition when exogenous noradrenaline is applied against a background of TNS and vice versa. (2) The possible potentiation by exogenous noradrenaline of the contractile effects of TNS as observed by Su(229) and Sjostrand et al(230), and demonstrated in the canine saphenous vein in Protocol Four of the present study. (3) The effects due to the relaxatory response to TNS observed in Protocol 3.1.

The findings and conclusions from the present investigation can be summarised as follows.

1. A background TNS contraction inhibited the contraction produced by exogenous noradrenaline in canine saphenous veins.
2. This inhibitory effect appeared to be greater with increasing magnitude of background TNS.
3. The inhibitory effect was specific for background TNS, as it was not observed with background contractions produced by tyramine (an indirectly acting sympathomimetic agent), methoxamine, phenylephrine and histamine.

4. The inhibitory effect was not antagonised by propranalol, cimetidine, aminophylline or indomethacin. Thus, the inhibitory effect is unlikely to be mediated by a β-receptor, a H_2-receptor, a P_1-purinoceptor or a prostaglandin metabolite of the cyclo-oxygenase pathway.
5. Protocol 1.1 and Protocol 1.2 carried out in a superfused preparation (in Protocol Two) demonstrated that exogenous noradrenaline inhibited the TNS induced efflux of total radioactivity and intact ^{3}H-noradrenaline during both protocols.
6. Following sympathetic blockade, TNS elicited a frequency dependent relaxation. This relaxation was observed in the presence of propranolol and atropine, i.e., it was non-adrenergic and non-cholinergic in nature.
7. This relaxatory response to TNS was resistant to tetrodotoxin but was abolished following cold storage of the saphenous veins. The relaxatory response was not affected by chemical sympathectomy with 6-hydroxydopamine.
8. The relaxatory response was not blocked by cimetidine, aminophylline, indomethacin, ascorbic acid or catalase. It was abolished by ouabain and zero-K^+ Krebs buffer solution suggesting that a functional Na^+/K^+ membrane pump was necessary for the observed relaxation.
9. Thus, in the canine saphenous vein TNS elicits a non-adrenergic, non-cholinergic relaxation, possibly mediated by a tetrodotoxin resistant nerve.
10. This non-adrenergic, non-cholinergic relaxation to TNS can be put forward as a possible explanation for the inhibition of exogenous

noradrenaline mediated contraction by background TNS observed in Protocol 1.1. However, other factors are also likely to be responsible for the findings in Protcol 1.1 and Protocol 1.2.

11. The contractile response to TNS in canine saphenous veins was potentiated by background exogenous noradrenaline induced contractions.
12. Low (sub-threshold) concentrations of exogenous noradrenaline elicited a potentiation of the responses to trains of TNS in the canine saphenous vein. If this pehnomenon is present with higher concentrations of exogenous noradrenaline, it could account for the findings in Protocol 1.2. i.e. the enhancement of the response to TNS by background contractions produced by exogenous noradrenaline.
13. There appears to be a significant interaction between the effects of exogenous noradrenaline and the effects of transmural nerve stimulation in isolated canine saphenous veins.

Considerations for the future

A number of findings from the present study merit further investigation. Firstly, no definite conclusion was arrived at, as to the origin of the relaxatory response observed in Protocol Three. Definite proof for a non-adrenergic, non-cholinergic intramural nerve, resistant to tetrodotoxin could be obtained if the relaxatory response could be demonstrated while stimulating the nerve trunk supplying the blood vessel proximally instead of applying transmural nerve stimulation. The canine lateral spahenous vein derives its adrenergic innervation from the upper lumbar roots via the lumbar sympathetic trunk. If the postulated relaxatory innervation also takes the same anatomical pathway stimulation at a proximal site may indeed be possible. Thus, an experiment could be carried out in vivo on an isolated, perfused, saphenous vein segment to simulate Protocol Three. The contractile response to the stimulation of the lumbar sympathetic nerves would have to be blocked using guanethidine and phenoxybenzamine. Following this blockade the vein could be contracted with prostaglandin $F_2\alpha$ and the response to lumbar sympathetic nerve stimulation elicited with the venous segment perfused at constanct flow with Krebs buffer solution while measuring the hydrostatic pressure inside the vein. The biggest problem in this protocol would be the isolation of the perfused venous segment from the rest of the circulation without damaging its innervation to any significant extent. This isolation from the rest of the circulation would be essential to avoid any secondary passive changes in pressure from the concomitant arteriolar constriction during the lumbar sympathetic stimulation.

Another approach that can be utilised to determine the origin of the relaxatory response in Protocol Three would be the morphological identification of non-adrenergic, non-cholinergic nerves in the saphenous vein. Electron-microscopy as well as immuno-histochemistry can be adapted for this purpose. The latter method using antibodies to vasoactive intestinal polypeptide and substance P has demonstrated the presence of these substances in nerves in a number of blood vessels.

The second question that has to be resolved is the mechanism behind the potentiation of the response to TNS by exogenous noradrenaline in Protocol Four. Recent experiments (in this laboratory) have demonstrated a similar potentiation of the response to TNS with methoxamine (selective α_1-receptor agonist), clonidine (relatively selective α_2-agonist) and adrenaline. The potentiation produced by the sub-threshold concentrations of noradrenaline and adrenaline may suggest a role for circulating catecholamines in vivo in enhancing the responses to sympathetic nerve activity. At present circulating catecholamines are not believed to exert any direct action on vascular smooth muscle as their plasma concentrations are well below the threshold for activation of vascular smooth muscle in vitro. Thus the investigation of the findings in Protocol Four, with a view to elucidating the mechanism behind the potentiation would be useful.

Thirdly, the ability of the calcium antagonist, diltiazem to block the contractile response to TNS while leaving the response to exogenous noradrenaline relatively unaltered is also interesting and deserves further investigation.

In summary, the results obtained in the present study may pave the way for a number of diverse investigations.

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Appendix I

Drugs and Chemicals

The Krebs buffer solution used, had the following composition (mmol/l) NaCl 116.0, KCl 5.4, $CaCl_2$ 1.2, $NaHCO_3$ 22.0, NaH_2PO_4 1.2, $MgCl_2$ $\cdot 6H_2O$ 1.2, glucose 10.1, $CaNa_2EDTA$ 0.023.

Sources of drugs used

1. aluminum oxide (Woelm Pharma-GmbH & Co., Eschwege, West Germany)
2. aminophylline: MW 420.4 (Abbot Laboratories Ltd., Montreal, Canada).
3. ascorbic acid: MW 176.1 (Sigma Chemical Co., St. Louis, U.S.A.)
4. atropine: MW 289.4 (Sigma Chemical Co., St. Louis, U.S.A.)
5. catalase (Sigma Chemical Co., St. Louis, U.S.A.)
6. cimetidine hydrochloride: MW 288.8 (Sigma Chemical Co., St. Louis, U.S.A.).
7. cocaine hydrochloride: MW 339.8 (Sigma Chemical Co., St. Louis, U.S.A.).
8. 3,4-dihydroxymandelic acid (DOMA):MW 184.1 (Sigma Chemical Co., St. Louis, U.S.A.)
9. 3.4-dihydroxyphenylglycol (DOPEG):MW 170.2 (Sigma Chemical Co., St. Louis, U.S.A.).
10. diltiazem hydrochloride:MW 451.0 (Sigma Chemical Co., St. Louis, U.S.A.).

11. di sodium ethylene diamine tetra-acetic acid:MW 336.2 (Sigma Chemical Co., St. Louis, U.S.A.).

12. Dowex-1 (Sigma Chemical Co., St. Louis, U.S.A.).

13. Dowex-50 (Bio-Rad Laboratories, Richmond, California, U.S.A.).

14. Guanethidine sulphate:MW 294.4 (Ciba-Geigy Canada Ltd.).

15. Histamine dihydrochloride:MW 184.1 (Sigma Chemical Co., St. Louis, U.S.A.).

16. 6-hydroxydopamine hydrochloride:MW 205.7 (Sigma Chemical Co., St. Louis, U.S.A.).

17. Indomethacin hydrochloride:MW 357.8 (Sigma Chemical Co., St. Louis, U.S.A.).

18. Methoxamine hydrochloride (vasoxyl):MW 247.7 (Burroughs Wellcome Ltd., Montreal, Canada).

19. 3-Methoxy-4hydroxymandelic acid (VMA):MW 198.2 (Sigma Chemical Co., St. Louis, U.S.A.).

20. 3-Methoxy-4hydroxyphenylgycol (MOPEG):MW 227.3 (Sigma Chemical Co., St. Louis, U.S.A.).

21. Noradrenaline bitartarate:MW 319.3 (Sigma Chemical Co., St. Louis, U.S.A.).

22. 7-^{3}H-noradrenaline (specific activity 10-30/mmol) (New England Nuclear, Boston, U.S.A.).

23. Normetanephrine hydrochloride (NMN):MW 219.7 (Sigma Chemical Co., St. Louis, U.S.A.).

24. Ouabain (octahydrate):MW 728.6 (Sigma Chemical Co., St. Louis, U.S.A.).

25. Octyl phenoxy polethoxyethanol (Triton X-100) (Sigma Chemical Co., St. Louis, U.S.A.).

26. Pentobarbital sodium (Somnotol): (M.T.C. Pharmaceuticals, Hamilton, Canada).

27. Phenoxybenzamine hydrochloride:MW 340.3 (Smith Kline and French Canada Ltd.

28. Phenylephrine hydrochloride:MW 203.7 (Sigma Chemical Co., St. Louis, U.S.A.).

29. Propranolol hydrochloride:MW 295.8 (Sigma Chemical Co., St. Louis, U.S.A.).

30. Prostaglandin $F_{2\alpha}$:(Prostin $F_{2\alpha}$, Dinoprost tromethamine) MW 475.6 (Upjohn Company of Canada, Don Mills, Canada

31. Tetrodotoxin:MW 319 (Sigma Chemical Co., St. Louis, U.S.A.).

32. Tyramine hydrochloride:MW 173.6 (Sigma Chemical Co., St. Louis, U.S.A.).

The concentrated stock solutions (10^{-4} mol/l in most cases) of the drugs were prepared in distilled water. 1.0 mg of sodium metabisulphite was added to each 10 ml of the stock solutions to minimise oxidation in the case of easily oxidisable drugs. Suitable dilutions of the drugs were prepared in Krebs buffer solutions each day.

Indomethacin was dissolved in equimolar Na_2CO_3 solution. Phenoxybenzamine was dissolved in propylene glycol. All drugs were added in 20-100μl aliquot to the tissue bath to produce the desired concentration in the bath fluid. Continuous bubbling of the tissue bath fluid with 95 per cent O_2-5 per cent CO_2 ensured that this desired concentration in the fluid was achieved almost instantaneously. The highest concentration of the drugs during dose-response curves were maintained in the tissue bath for as short a duration as possible to minimise desensitization. Drugs were removed from the bath fluid by repeated rinsing of the bath with fresh Krebs buffer solution. When a drug had to be kept in the tissue bath at a fixed concentration throughout an experiment (e.g., propranolol and atropine in Protocol 3.1) the drugs were added to the reservoir of Krebs solution feeding the baths. This prevented the repeated additions of small aliquots of the drugs that would otherwise be necesary (thus minimising the error) during each rinse of the tissue bath.

Appendix II

Solutions used for column chromatographic analysis

1. 2 litres 2N hydrochloric acid
    332 ml of 12N HCl
    400 ml of 0.5% Triton X-100
    1268 ml H_2O

2. 1 litre sodium phosphate buffer pH 6.5
    6.6 g KH_2PO_4
    2.67 g Na_2HPO_4
    1 g Na_2EDTA

    Make up to 1ℓ with 0.1% Triton x 100: pH checked and titrated to 6.5 if necessary.

3. 1 litre 6N HCl/ethanol
    250 ml 12N HCl
    500 ml ethanol
    240 ml H_2O (no Triton X-100)

4. 1 litre 0.2N hydrochloric acid
    100 ml 2N HCl
    200 ml 0.5% Triton X-100
    700 ml H_2O

5. 1 litre 1 N hydrochloric acid
    500 ml 2N HCl
    200 ml 0.5% Triton X-100
    300 ml H_2O

6. 1 litre 0.2N acetic acid
    200 ml 1 N acetic acid
    200 ml 0.5% Triton X-100
    700 ml H_2O

7. 1 litre 0.1% Triton X-100
    200 ml 0.5% Triton X-100
    800 ml H_2O

Appendix III

Solutions used during chemical sympathectomy with 6-hydroxydopamine

Composition of unbuffered physiological salt solution used (mmol/l): NaCl 136.7, $MgCl_2$ 2.1, KCl 2.7, $CaCl_2$ 1.8, glucose 5.4, $CaNa_2EDTA$ 0.023.

The pH of the solution was adjusted to 4.9 using reduced glutathione. 6.0 mg of 6-hydroxydopamine was dissolved in 20 ml (1.46×10^{-3} mol/l) of this physiological salt solution just prior to use on each day.

Appendix IV

The definitions of some frequently used terms in Pharmacology are given below. The definitions do not exactly correspond to the correct meaning of these terms in the English language, nevertheless, these definitions have become irrevocably embedded in Pharmacological literature.

Washing, rinsing:	the replacement of the bath fluid with fresh Krebs buffer solution containing no drugs (unless specified otherwise).
Dose:	the aliquot of a drug added into the tissue bath to produce a desired concentration in the bath fluid following the dilution of the drug in the bath.
Dose-reponse curve:	refers to the effect to an agonist (y axis) plotted against the concentration of the agonist (x axis) i.e. concentration effect curve.
Incubation:	Exposing a tissue to a desired concentration of a drug for a specified length of time. This is commonly used in reference to antagonists which are kept in contact with a tissue for a length of time before repetition of the agonists effects.